The Role of the Inflammasome in Skin Inflammatory Diseases and Its Regulation in General Processes of Inflammation

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Zusammenfassung

Interleukin-1β (IL-1β) ist ein entzündungsförderndes Zytokin, welches eine Schlüsselrolle in akuten und chronischen Entzündungen spielt. Insbesondere seine Rolle in der Pathogenese der Autoinflammation machen IL-1β zu einem idealen Ziel um die schwere verschiedenartigster Erkrankungen schnell zu reduzieren. IL-1β wird in seiner biologisch inaktiven Pro-Form produziert und um aktiviert und sekretiert zu werden muss es durch einen Multiproteinkomplex, das sogenannte Inflammasom, geschnitten werden. Daher spielt die Aktivität des Inflammasoms eine wichtige Rolle in vielen pathologischen und physiologischen Prozessen.

Der Hauptteil meiner Doktorarbeit befasst sich mit der Identifikation der Mechanismen, welche das Inflammasom regulieren könnten. Unter den verschiedenen zur Zeit bekannten Inflammasomen ist das am meisten studierte das NLRP3 Inflammasom. Es konnte gezeigt werden, dass viele verschiedene Stimulantien mit unterschiedlichen Eigenschaften (Umweltstress, pathogene Partikel, pathogen-assoziierte Strukturen, gefahr-induzierende Metabolite, gefahr-induzierende Strukturen) das NLRP3 Inflammasom aktivieren können. Daher muss der Zelle ein gemeinsamer Pfad zugrunde liegen, welcher die unterschiedlichen Stimuli in einen einzelnen Pfad bündelt, welcher wiederum zur Aktivierung des Inflammasoms führt. In der vorliegenden Arbeit konnten wir zeigen, das Mitogen-Associated Protein Kinasen (MAPKn) aktiviert werden nachdem Immun- und Nicht-Immunzellen verschiedensten Stimulatoren des Inflammasoms ausgesetzt wurden und das diese Stimulatoren beteiligt sind an der Schädigung der Mitochondrien und dem Auslaufen mitochondrialer DNA in das Zytosol, was im Anschluss zur Aktivierung des NLRP3 Inflammasoms führt. Dieser Prozess ist auf die zwei BCL-2 Familienmitglieder, BAD und BCL-XL angewiesen und wird durch MAPK und Phosphatase-1 abgestimmt.

Acne vulgaris ist eine der am Häufigsten auftretenden Hauterkrankung welche Rund 80% der Bevölkerung irgendwann in ihrem Leben betrifft. Es ist eine multifaktorielle Erkrankung, welche das Potential in sich birgt sich in einer schweren Form zu manifestieren. In dieser Doktorarbeit zeigen wir, dass *Propionibacterium acnes*, ein Bakterium welches sich in entzündeten Aknelesionen (Pusteln) findet, fähig ist, nach Internalisierung, das NLRP3 Inflammasom zu aktivieren, was zur Freisetzung von cathepsin B-abhängigen reaktiven Sauerstoffradikalen führt. Wir konnten weiter zeigen, dass das NLRP3 Inflammasom in myeloiden Zellen eine wichtige Rolle in einem *in vivo* Modell spielt und dass das gezielte Ansteuern des IL-1β ein effektiver Ansatz ist neutrophile Entzündungsreaktionen zu hemmen.

Kontakt-Überempfindlichkeit ist ebenfalls eine häufige Hauterkrankung in welcher IL-1β eine wichtige Rolle spielt. Kontaktallergene sind chemische kleine organische Moleküle welche an körpereigene Proteine binden und so ein Neo-Antigen generieren, in einem Prozess welcher als Haptenbildung bezeichnet wird. Haptene, wie Dinitrofluorobenzen (DNFB) und Dinitrothiocyanobenzen (DNTB) präsentieren kreuzreaktive Antigene, welche sensibilisierende oder tolerierende T-Zellantworten hervorrufen. In dieser Doktorarbeit konnten wir im Mausmodell zeigen, dass das NLRP3 Inflammasom zur Sensibilisierung gegen DNTB und zur Tolerierung von DNFB führen kann, was den ersten Beweis anführt, dass das Inflammasom eine Antigen-spezifische erworbene Immunantwort definieren kann und neue Strategien zur Beeinflussung von T-Zellantworten im Lebenden andeutet.

Zusammenfassend geben die Ergebnisse dieser Doktorarbeit einen Einblick in die Regelung der Aktivierung des Inflammasoms und die daraus resultierende IL-1β Ausschüttung durch MAPKn und enträtseln die entscheidende Rolle des NLRP3 Inflammasoms in der Pathogenese der Acne vulgaris und der Entstehung der Kontakt-Überempfindlichkeit.

Summary

Interleukin-1β (IL-1β) is a key pro-inflammatory cytokine associated to acute and chronic inflammation. It plays a crucial role in the pathogenesis of auto-inflammatory diseases and targeting IL-1β is an efficient way to obtain a sustained and rapid reduction in the severity of several disorders. IL-1β is synthetized in a pro-form, which is biologically inactive, and in order to become active and secreted needs to be cleaved by cytosolic multiprotein complexes called inflammasomes. Therefore, regulation of inflammasome activity is crucial in many pathological and physiological conditions.

In the main part of this thesis, we looked for the possible mechanisms that could be responsible for the regulation of the inflammasome. Among the different inflammasomes known to date, the most studied one is NLRP3 inflammasome. Many different stimuli of different natures (environmental stresses, pathogen particles, pathogen-associated molecular patterns, metabolites considered as danger signals, danger-associated molecular patterns) have been shown to activate the NLRP3 inflammasome. Therefore its hypothesized that there may be a common pathway used by the cell to centralize such diverse stimuli to a unique pathway leading to inflammasome activation. In the present work, we could demonstrate that Mitogen-Associated Protein Kinases (MAPKs) become active upon stimulation of immune and non-immune cells with several inflammasome activators and that they are involved in mitochondria damage and leakage of mitochondrial DNA to the cytosol, subsequently activating the NLRP3 inflammasome. This process is dependent on two BCL-2 family members, namely BAD and BCL-XL and modulated by MAPK phosphatase-1.

Acne vulgaris is the most common skin disease, affecting around 80% of the population at some time in their lives. It is a multifactorial inflammatory disease, which can potentially manifest in very severe forms. In this thesis, we

show that Propionibacterium acnes (P.acnes), a bacterium found in inflammatory acne lesions (pustules), is able to activate the NLRP3 inflammasome upon its internalization, leading to reactive oxygen species generation and depending on cathepsin B. We could also show that NRLP3 inflammasome in myeloid cells plays a key role in an *in vivo* model, and that targeting IL-1β is an effective approach to prevent neutrophilic inflammation induced by P.acnes.

Contact hypersensitivity is also a common skin disorder in which IL-1β was shown to play a role. Contact allergens are chemically reactive small organic molecules binding to self-proteins to generate immunogenic neo-antigens, through a process termed haptenization. Haptens such as dinitrofluorobenzene (DNFB) and dinitrothiocyanobenzene (DNTB) provide cross-reactive antigens driving opposite, sensitizing versus tolerizing, T cell responses. In this thesis we show that, in mice, the NLRP3 inflammasome can turn DNTB into a sensitizer and DNFB into a tolerizer, providing the first evidence that the inflammasome can define the type of adaptive immune response elicited by an antigen, and hint at new strategies to modulate T cell responses *in vivo*.

Taken together, the results of this thesis provide insight to the regulation of inflammasome activation and subsequent IL-1β secretion by MAPK, they also unravel the crucial role of the NLRP3 inflammasome in the pathogenesis of Acne and in the process of sensitization of molecules in Contact Hypersensitivity.

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Contents

Chapter 1

Introduction

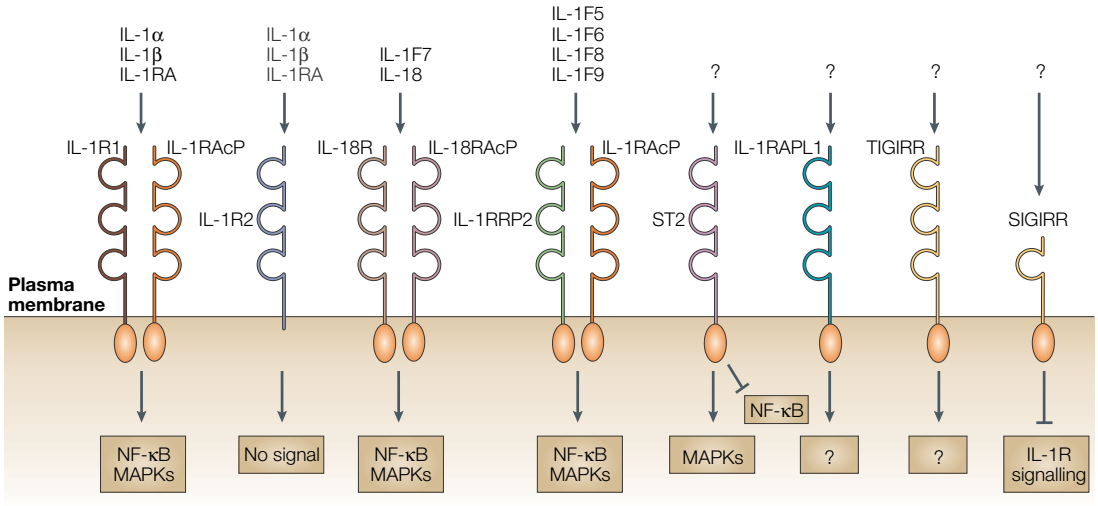
1.1 Interleukin 1β

Interleukin 1 (IL-1), the first discovered cytokine, by Gery et al. in 1972[1] was, at that time, called lymphocyte activating factor (LAF) due to its lymphocyte mitogen activity. Since then it has been shown to have several other pro-inflammatory properties, as fever, hypotension, tachycardia, hyperinsulinemia, neutrophilia, increased antibody production, inhibition of tolerance to protein antigens[2] and dendritic cell maturation[3]. The cytokine receptor for IL-1 was characterized; sister cytokines were discovered (IL-1α and β, IL-1RA, IL-18) and a whole mechanism of sensing danger by innate immunity (inflammasomes) was unravel.

The IL-1 family consists of several cytokines, which bind to receptors sharing homology with Toll-like receptors (TLRs), and include 10 members (IL-1R1-10) (Figure 1).

IL-1α and IL-1β share 27% homology, are structurally quite similar, and are biologically equivalent, with very minor functional differences. IL-1s are pleiotropic factors that act in local and systemic inflammation.

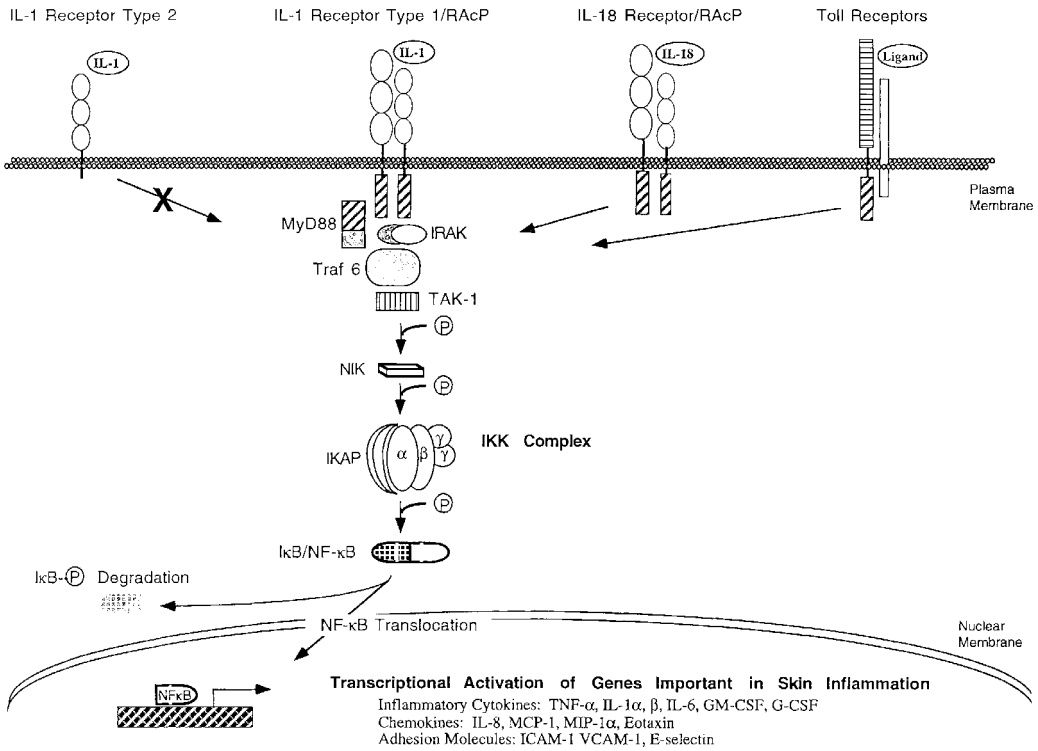
IL-1 cytokines exert their biological functions through binding to their receptors. The receptor complex for IL-1α and IL-1β consists of IL-1R1 and the IL-1R accessory protein (IL-1R-AcP)[4]. Upon dimerization, the complex activates an intracellular signaling cascade through the toll-domains in the cytoplasmic part, which results in activation of NF-kB and to a certain extent Mitogen Activated Protein (MAP) kinases.



adapted from Allan et al., 2005[5]

Figure 1. The interleukin-1 family. The various members of the interleukin-1 (IL-1) family and their receptors are shown. Possible receptor–ligand interactions that initiate downstream signaling are also indicated. IL-1F, IL-1 family; IL-1R1, type I IL-1 receptor; IL-1R2, type II IL-1 receptor; IL-1RA, IL-1-receptor antagonist; IL-1RAcP, IL-1-receptor accessory protein; IL-1RAPL1, IL-1-receptor accessory-protein-like 1; IL-1RRP2, IL-1-receptor-related protein 2; IL-18R, IL-18 receptor; IL-18RAcP, IL-18-receptor accessory protein; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; SIGIRR, single immunoglobulin IL-1-receptor-related molecule; TIGIRR, three-immunoglobulin-domain-containing IL-1-receptor-related molecule.

These events lead to expression of genes encoding mainly pro-inflammatory proteins such as IL-6, IL-8 and TNF-α (figure 2). The antagonist of IL-1 is IL-1 receptor antagonist (IL-1 Ra), that can also bind to IL-1R1 but lacks the ability to recruit IL-1R AcP. Therefore, IL-1 Ra prevents IL-1 signaling by competing with IL-1α and IL-1β. The second IL-1 receptor (IL-1R2) lacks an intracellular domain for signal transduction and is not able to dimerize with IL-1R AcP, therefore acting as a decoy receptor. Furthermore, a truncated soluble form of this receptor (sIL-1R2) can be secreted by cells. sIL-1R2 also traps IL-1 and IL-1R AcP molecules.



adapted from Murphy et al., 2000[6]

Figure 2. IL-1 induces the transcription of genes involved in inflammation via activation of the transcription factor NF-kB. The membrane-bound IL-1R type 1 can bind either IL-1α or β and, when complexed to the receptor accessory protein, will initiate the signal transduction cascade that ultimately results in stimulation of transcription of adhesion molecules, cytokines, and pro-inflammatory genes. This complex recruits the adaptor molecule MyD88 that binds to the IRAKs. The IRAKs bind to another adaptor molecule, TNF receptor associated factor 6, which associates with TAK1, the kinase that phosphorylates the NF-kB-inducing kinase. NF-kB-inducing kinase activates IKK, which can phosphorylate IkB, causing its rapid degradation. NF-kB is then free to migrate into the nucleus, where it binds to specific sequences (kB sites) in the promoter region of multiple inflammatory genes.

1.2 Regulation of IL-1 production

Since IL-1 is such a potent and important cytokine it is not surprising that the generation of IL-1 activity is tightly regulated at different levels. Evidence suggests that the expression of IL-1α although regulated, exists to some extent at a constitutive level, whereas the expression of IL-1β is only detectable in most cell types upon stimulation [7, 8]. Expression of IL-1a and b is induced by activation of the transcription factor NF-κB, which can be induced by bacterial endotoxins, viruses, mitogens, antigens, cytokines like TNF-α, IFN-α, IFN-β and IFN-γ, as well by IL-1 itself. In addition, it has been shown that IL-1β mRNA can be very unstable [9, 10].

Both IL-1α and IL-1β are expressed as 31 kDa pro-molecules that lack a signal peptide for ER/Golgi-dependent secretion. IL-1α and IL-1β are

maturated by the protease caspase-1 [11], which is required to generate the active forms of the cytokines. Although extracellular pro-IL-1β can be cleaved after cell disruption by other proteases including elastase, cathepsin G, collagenase and protease 3, which are released at sites of inflammation [12, 13] caspase-1 is the major intracellular activator of IL-1β. In contrast to proIL-1β, proIL-1α is able to bind and activate IL-1RI although proteolysis of its prosequence can enhance its biological activity [14]. Therefore, cell lysis upon injury or trauma generates IL-1 activity due to passive release of proIL-1α, whereas passively released proIL-1β does not possess biological activity.

Most secreted proteins contain a signal peptide at the amino terminus, which directs them through the classical endoplasmic reticulum/Golgi-dependent secretion pathway to the extracellular space. In contrast, proIL-1α, proIL-1β and several other proteins are leaderless, thus lacking a signal peptide, and are secreted by a non-canonical pathway, called the unconventional protein secretion pathway [15]. The mechanism and regulation of unconventional secretion is only poorly understood but it has been recently demonstrated that caspase-1 activity is required for the secretion of leaderless proteins [16, 17].

IL-1Ra blocks IL-1 activity. Although IL-1Ra, IL-1α and IL-1β have a similar affinity to IL-1RI, a large excess (100-1000) of IL-1Ra over IL-1 is required for efficient blockade of receptor signaling *in vitro* and *in vivo* [18].

The regulation of caspase-1 activity is done by inflammasomes, discussed later in this introduction.

IL-1β plays a key role in rheumatoid arthritis, sepsis, diabetes, sunburn, contact hypersensitivity, graft-versus-host disease, anti-cancer response, adjuvant chemotherapy effectiveness, hypertension, diabetes, Alzheimer's disease, and some auto-inflammatory diseases like Familial Mediterranean fever, Familial cold autoinflammatory syndrome (FCAS), Muckle-wells syndrome, Neonatal-onset multi-inflammatory disease (NOMID), Mevalonic aciduria, HyperIgD syndrome, Adult-onset Still's disease, Schnitzler's syndrome, Behcet's syndrome, PAPA syndrome, CINCA and urate crytal arthritis (gout)[7, 19-29].

Therefore, understanding more about IL-1β and its mechanisms of regulation, has a direct relevance for our understanding of these diseases and potentially for their management.

1.3 The inflammasomes

Despite the fact that IL-1 was the first cytokine discovered, the mechanism that leads to its secretion has only been relatively recently discovered. Named the inflammasomes and first discovered by Tschopp and colleagues[30] in 2002, the inflammasomes are high-molecular-weight protein complexes that are localized in the cytosol and serve as platforms for the recruitment and autoproteolytic activation of certain caspases, notably caspase-1 (Figure 3).

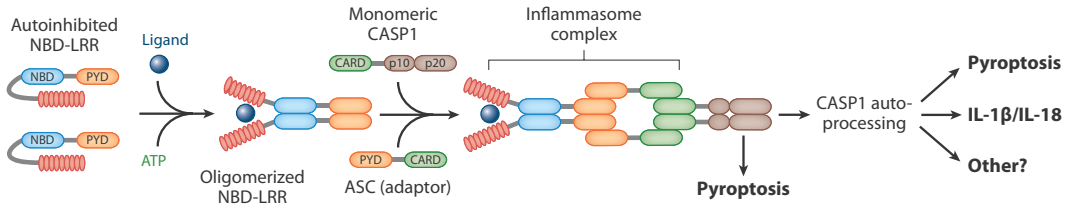


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Figure 3: Model of inflammasome activation. Cytosolic nucleotide-binding domain, leucine-rich repeat (NBD-LRR) proteins are maintained in an autoinhibited state until ligand recognition and ATP binding drive oligomerization via the NBD domain. Recruitment of CASP1 to form the inflammasome complex occurs directly via CARD-CARD interactions or indirectly via the adaptor ASC. Monomeric CASP1 is believed to be activated by proximity-induced autoproteolysis (autoprocessing), leading to downstream effector functions such as pyroptosis and processing of pro-IL-1β and pro-IL-18. Abbreviations: CARD, caspase activation and recruitment domain; PYD, PYRIN domain.

Caspases 1, 4, and 5, along with caspase 11 and caspase 12 in the mouse, are called inflammatory caspases and are distinct from the caspases involved in the induction of apoptosis. Caspase-1 is the best characterized among inflammatory caspases and was first described as the protease required for the proteolytic cleavage of pro-IL-1β and pro-IL-18, resulting in their functional maturation and release[31, 32] .

In the last few years, it has become clear that there are in fact multiple distinct inflammasomes, each of them is activated by a unique stimulus or a

group of stimuli that can include infectious agents as well as noninfectious stimuli. The formation of each inflammasome is dictated by a unique scaffolding protein (Figure 4).

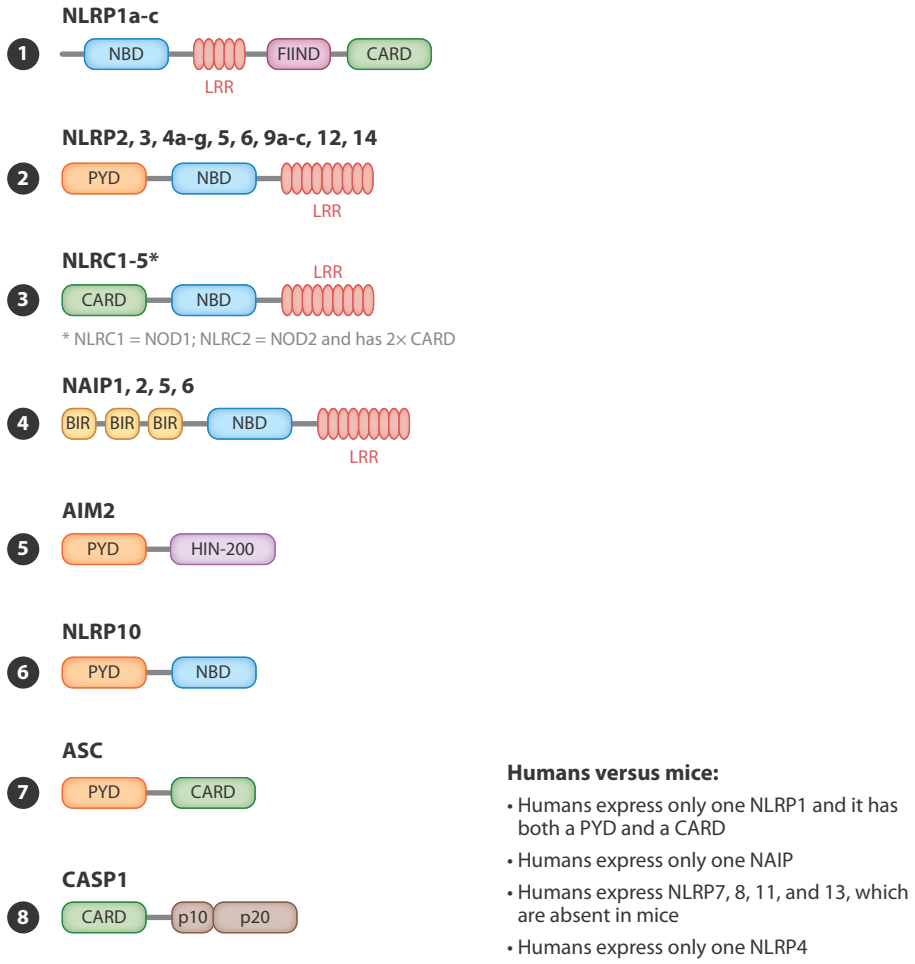

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Figure 4. Inflammasome proteins and their domains. NBD-LRR family members can be categorized according to the unique domains they encode: 1. NLRP1: FIIND; 2. all other NLRP proteins: PYRIN domain (PYD);3. NLRC: CARD; 4. NAIP: BIR domains.5. AIM2 belongs to the PYHIN family of proteins and uses the HIN-200 domain for ligand recognition.6. NLRP10 is the only family member lacking an LRR domain. 7. ASC is an adaptor required by NLRP proteins for recruitment of CASP1. 8. Caspase-1 contains a CARD and a protease domain that autoprocesses into p10 and p20 subunits. CASP11 and CASP12 are additional inflammatory caspases in mice; CASP4 and CASP5 are additional inflammatory caspases in humans.

Most of these scaffolding proteins contain a nucleotide-binding domain (NBD) and leucine-rich repeats (LRRs) and are members of the NBD-LRR (NLR) superfamily. The LRRs are believed to have two functions. First, LRRs maintain NLRs in an auto-inhibited state, because deletion of LRRs generally

leads to a constitutively active NLR. Second, by analogy to the well-characterized ligand binding function of LRR domains in Toll-like receptors (TLRs), the LRRs of NLRs are believed to mediate recognition of pathogen-derived (or potentially self-derived) ligands. The NBD is believed to mediate the assembly of NLRs into an oligomerized state that is critical for the induction of downstream signaling. In NLRs, signaling is initiated by CARDs (caspase activation and recruitment domains) that can recruit Caspase-1 directly or by PYRIN domains that recruit caspase-1 via the CARD-PYRIN-containing adaptor protein ASC. Once recruited to an oligomerized inflammasome, caspase-1 is activated via dimerization and auto-proteolysis. Interestingly, non-NLR proteins such as PYRIN-HIN-200 (PYHIN) proteins have also been shown to mediate caspase-1 activation[32-36]. Although PYHIN proteins lack NBDs and LRRs, they may nevertheless exhibit the properties of auto-inhibition and ligand-induced oligomerization[37].

Four types of inflammasome complexes that are activated upon encounter with distinct types of DAMPs and/or PAMPs have been characterized. These are the Aim2, NLRP1, NLRP3 and NLRC4 inflammasomes. The Aim2 inflammasome is activated by binding to viral and bacterial double-stranded DNA resulting from intracellular pathogens, the NLRP1 inflammasome by muramyl dipeptide, and NLRC4 inflammasome by flagellin [38]. The NLRP3 inflammasome is most likely the most important inflammasome since it assembles in response to a large variety of PAMPs and DAMPs, and its deficiency is already clearly associated with immunological dysfunction in mice as well as disease states in men. Since a direct interaction of any of these activators with NLRP3 could not be shown, indirect activation mechanisms have been suggested. Particulates such as asbestos or crystals such as gout-causing monosodium urate (MSU) or cholesterol are phagocytosed by macrophages [39, 40]. However, their clearance is not efficient and this results in lysosomal rupture, which, in turn, activates the NLRP3 inflammasome by an unknown mechanism perhaps involving cathepsins [40, 41]. As all NLRP3 activators induce the generation of reactive oxygen species (ROS), it has been reported that this ROS production triggers NLRP inflammasome activation [41]. Generation of ROS

induces the dissociation of thioredoxin from thioredoxin-interacting protein. Then the latter is able to bind NLRP3, which induces inflammasome activation [42]. The predominant source of ROS generated by danger signals is most likely the mitochondria, which also controls inflammasome activation via the release of mitochondrial DNA [43, 44].

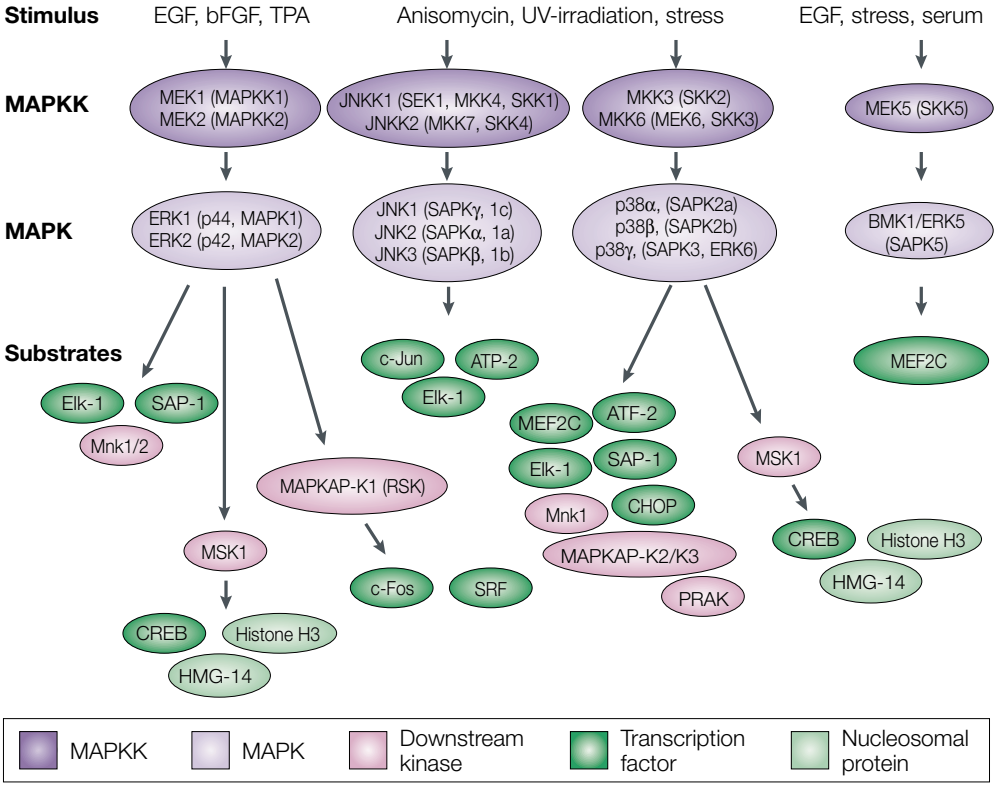
It has become clear that inflammasomes not only have diverse mechanisms of activation but can also have diverse functions that extend beyond cytokine processing. Most notably, inflammasome activation is frequently associated with a rapid and lytic form of host cell suicide called pyroptosis[45]. Inflammasome activation plays a critical role in mediating host defense, but inappropriate or excessive inflammasome activation can also be detrimental and lead to pathological situations. Inflammasomes constitute a complex and surprisingly diverse set of cytosolic sensors that must be strictly regulated.

1.4 MAPK kinases

Mitogen-activated protein kinase (MAPK) signal transduction pathways are ubiquitous and highly evolutionarily conserved mechanisms of eukaryotic cell regulation. The multiple MAPK pathways present in all eukaryotic cells enable coordinated and integrated responses to diverse stimuli. These stimuli include hormones, growth factors, cytokines, agents that act through G protein-coupled receptors, transforming growth factor (TGF)-β-related agents that act through Serine-Threonine kinase receptors, pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) that recruit pattern recognition receptors (PRRs), and environmental stresses. MAPK pathways, once activated, exert major effects on cell physiology. MAPKs orchestrate gene transcription, protein biosynthesis, cell cycle control, apoptosis, and differentiation[46-54]. Their targets have important implications in organ development, innate and acquired immunity,

and diseases such as atherosclerosis, hypertension, tumorigenesis, and type 2 diabetes [55].

Mammals express multiple MAPK pathways. The majority of these are, along with the nuclear factor-κB (NF-κB) pathway, activated by stress and inflammatory stimuli. MAPK pathways consist principally of 3 distinguished pathways: JNK, ERK and P38. An overview of MAPK pathways is shown in figure 5.



adapted from Hazzalin et al., 2002

Figure 5. Mammalian MAPK cascades**.** Schematic representation of mammalian MAPK cascades and target substrates, including transcription factors that are involved in the regulation of *fos* and *jun* immediate-early (IE) genes. The four characterized MAPKs comprise the extracellular- signal regulated kinase/mitogen-activated protein kinase (ERK/MAPK), c-Jun amino-terminal kinase/stress-activated protein kinase (JNK/SAPK), p38/reactivating kinase (RK)/p40/cytokine-suppressive anti-inflammatory drug-binding protein (CSBP)/Mxi2 kinase and big MAPK (BMK/ERK5) subtypes. bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; MEK, MAPK/ERK kinase; MKK/MAPKK, MAPK kinase; SKK, SAPK kinase; TPA, 12-*O*- tetradecanoylphorbol-13-acetate.

1.4.1 MAPK pathways in innate immunity and TLR responses

Innate immunity represents the first line of defense against invading microbial pathogens. PRRs are known to have an important contribution in innate immune responses. PRRs include membrane-associated TLRs as well as cytosolic nucleotide oligomerization domain-leucine-rich repeat (NOD-LRR) and RIG-like helicase (RLH) proteins. These receptors, upon engagement with agonists, recruit a variety of adaptor proteins, including myeloid differentiation factor-88 (MyD88) and Toll/IL-1 receptor (TIR)-domain-containing adapter-inducing interferon-β (TRIF). [56-59].

PRRs are trigered both by PAMPs (molecular moieties present on invading pathogens), and by DAMPs (molecules produced endogenously in conditions of physiological stress). DAMPs with known relevance to disease include oxidized LDL (oxLDL), relevant to atherosclerosis and crystalline uric acid, relevant to gout. Specific PAMPs and DAMPs recruit distinct groups of PRRs. Notably, LPS from Gram-negative bacteria recruits TLR4, and bacterial peptidoglycans recruit TLR-2 and -6. TLR-2, -4, and -6 are also recruited by oxLDL [56-60].

Activation of PRRs by microbial pathogens leads to an inflammatory response, cytokine production, leukocyte activation, fever, and, in extreme cases systemic inflammation/infection, edema, shock and disseminated intravascular coagulation, events that require activation of MAPKs (ERK, JNK, and p38)[56-59].

1.4.2 ERK

The extracellular signal-regulated kinases (ERKs-1 and -2; MAPKs-3 and -1, respectively) were the first mammalian MAPKs to be identified. The ERKs are most familiar as insulin and mitogen-activated MAPKs recruited by agonists that engage the Ras proto-oncoprotein[61, 62].

In many instances, the ERKs can also be activated, in a manner independent of Ras, by proinflammatory stimuli including cytokines of the tumor necrosis factor (TNF) family, by PAMPs, such as LPS and by DAMPs, such as oxidized low-density lipoprotein (LDL), and crystalline uric acid. These mechanisms of ERK activation play an important role in innate immunity and inflammation.

1.4.3 JNK

The c-Jun NH_2-terminal kinases (JNKs – JNK-1, JNK-2 and JNK-3) were initially identified as a protein kinase activity purified from cycloheximide-treated rat liver. JNKs can be activated by mitogens and are also vigorously activated by a variety of environmental stresses (heat shock, ionizing radiation, oxidants), genotoxins (topoisomerase inhibitors and alkylating agents), ischemic reperfusion injury, mechanical shear stress, vasoactive peptides, proinflammatory cytokines, PAMPs/DAMPs and translational inhibitors such cycloheximide and anisomycin[63-65]. JNKs are also activated by tunicamycin, which leads to ER stress, implicating that JNKs are important effectors for the ER stress response. This has a direct implication in high-fat diet, which triggers ER stress *in vivo*, and this may play a critical role in triggering insulin resistance and high-fat diet-induced inflammation.

1.4.4 P38

P38 was originally identified as a stress- and IL-1-activated kinase that could phosphorylate and activate MAPK-activated protein kinase-activated protein kinase-1 (MK2; sect. IID1). MK2 is a member of a Ser/Thr kinase family that, among other substrates, phosphorylates and activates the small heat shock protein Hsp27[66, 67].

There are four p38 genes, namely p38α-δ, also called MAPK-14 (p38α), -11 (p38β), -12 (p38γ), and -13 (p38δ)[66-74], all of which are preferentially activated in situ by environmental stresses, inflammatory cytokines and PAMPs.

1.5 The skin

The skin plays a key role in protecting the body against external insults including pathogens and against excessive water loss. Its other functions are insulation, temperature regulation, sensation, and the production of vitamin D. Its outer layer is the epidermis, which consists mainly of keratinocytes, but also contains Langerhans cells, a specialized form of DCs (dentritic cells), and some pigment-producing melanocytes [75, 76]. Keratinocytes express several types of keratins, proteins, which are expressed only in epithelia, form intermediate filaments through assembly into bundles and generate the toughness of the epidermis [77]. In the underlying dermis several types of immune cells can be found such as macrophages, dermal dendritic cells, T cells and mast cells. They are embedded in connective tissue, which is made up by a mixture of extracellular matrix proteins produced by fibroblasts. The supply of nutrients to the skin is guaranteed by blood vessels in the dermis, the latter also containing lymphatic vessels and nerve endings. The epidermis is a constantly renewing tissue. Under normal conditions, proliferation of keratinocytes is restricted to the basal cell layer, where stem cells and transit amplifying cells are located. During an apoptosis-like process of terminal differentiation, keratinocytes change their expression profile and properties whilst migrating to the outer surface of the epidermis. This process of terminal differentiation generates densely packed layers with dead, flat and keratin-filled, most upper corneal layer keratinocytes called corneocytes at the surface, which form a protective envelope.

Under homeostatic conditions, the skin is colonized by a certain number and diversity of microorganisms on its surface. The dynamic equilibrium between the epidermis and microorganisms is regulated amongst others by sebocytes, which are located in sebaceous glands within the dermis and produce anti-microbial lipids, as well as by the microbes themselves, which produce antibiotic and antifungal substances as well as bacteriolytic enzymes. In addition, keratinocytes express antimicrobial substances or peptides constitutively and upon injury or infection, e.g. by stimulation of TLRs through PAMPs and DAMPs [78]. In addition to their antimicrobial properties,

certain keratinocyte-derived antimicrobial peptides as well as cytokines can influence the immunological properties of dendritic cells and T cells, which are also influenced by the stimulation of their own TLRs and can activate adaptive immunity [75, 76]. Given this, the skin has on one hand to ensure an efficient defense against pathogens and immunosurveillance, and on the other hand minimize excessive immune responses, which can result in disease states such as allergy, chronic inflammation and autoimmunity.

1.6 Acne vulgaris

Acne vulgaris - an inflammatory disease of the pilosebaceous unit - is the most common skin disease, affecting 60-70% of individuals at some time during their lives. Twenty percent of patients will have severe inflammatory acne that results in permanent scarring of the skin, and significant psychosocial effects. Acne lesions develop in the pilosebaceous unit as subclinical microcomedones that may evolve into closed or open comedones and inflammatory lesions such as papules, pustules, nodules and cysts. A severe inflammatory variant of acne, named acne fulminans, can be associated with fever, arthritis, and other systemic symptoms [79].

The pathogenesis of acne vulgaris is multifactorial, and remains to be completely elucidated. The main underlying cause of acne is genetic predisposition, the condition being inherited in an autosomal dominant pattern with incomplete penetrance. Other factors implicated in the development or severity of acne include excess sebum production and its consequences, hormonal factors mainly androgens, proliferation and retention of follicular keratinocytes resulting in follicular obstruction, the presence and activity of *Propionibacterium acnes (P. acnes hereafter),* and inflammation. The exact triggers for inflammation, and the molecular mechanisms involved are only partly elucidated however to date. One key factor that contributes to the inflammation observed in acne is *P. acnes*, a component of the normal skin flora, that can proliferate and be found in significantly increased numbers within the pilosebaceous units of patients with acne. The role of *P. acnes* as

an etiological factor in acne is well established. *P. acnes* is thought to contribute to the inflammatory nature of acne by inducing monocytes to secrete proinflammatory cytokines including IL-6, TNF-α, IL-1β and IL-8. In addition, *P. acnes* releases lipases, proteases and hyaluronidases that are considered to contribute to tissue injury. A direct link between *P. acnes,* the above-mentioned cytokines, and inflammation has never been made *in vivo,* and the exact molecular mechanism by which P.*acnes* induces Inflammation is unknown.

The molecular mechanism(s) by which *P. acnes* induces monocyte cytokine release is thought to involve pattern recognition receptors (PRRs) of the innate immune system - notably Toll-like receptors (TLR) 2 and 9. NFkB activation through TLR2 signalling has been shown to be necessary for the production of IL-6, IL-8 and IL-12 *in vitro* by antigen-presenting cells (APCs) upon *P. acnes* infection [80]. In addition, TLR9 has been shown to be required for TNF and IFN-γ production upon *P. acnes* infection *in vitro* [81]. Interestingly, TLR4 expression has also been shown to be increased in the epidermis of patients with acne lesions [82].

1.7 Contact Hypersensitivity

Allergic contact dermatitis is a common skin disease that is caused by type IV delayed-type hypersensitivity responses to antigens that come into contact with the skin[83]. Allergic contact dermatitis is a major cause of occupational skin disease, and it accounts for approximately 20% of all work-related health complaints. It results in an estimated 4 million lost work days and has an associated cost of almost US$400 million per year in the United States alone[84-86]. One of the clinically most important categories of contact allergens is small organic molecules that are chemically reactive (chemical sensitizers). They bind to self proteins to generate immunogenic neo-antigens, through a process termed haptenization. Contact allergens are common in cosmetics, personal care products and jewellery, as well as in the workplace, regardless of whether it is in an industrial, health care or office

setting. Examples of common allergens are inorganic chemicals, such as nickel, and organic chemicals, such as those found in fragrances and dyes[87].

There are a series of fundamental events that lead to immune recognition of the hapten–self-complex, the first is percutaneous penetration through the stratum corneum barrier, which is the water-impermeable outer layer of the skin. Compounds must be less than 500 daltons for efficient penetration[88]. This step allows the chemical sensitizer to come into contact with and haptenize self proteins in the skin. Haptens or haptenated self proteins are recognized amongst others by innate immune mechanisms in the skin, and this leads to the induction of several pro-inflammatory mediators, including interleukin-1β[89]. As a result, skin-resident dendritic cells (DCs) become activated. These DCs — which may have been directly haptenated or could have acquired haptenated proteins from their surroundings migrate to skin-draining lymph nodes, where they present peptides from haptenated proteins to activate memory and naive T cells. In the final step, hapten-induced inflammation recruits activated effector T cells back to the initial site of antigen encounter in the skin. The effector T cells release pro-inflammatory cytokines, such as interferon-γ, and promote the killing of haptenized cells, resulting in the development of the classic inflammatory rash that is seen in patients with allergic contact dermatits[88, 90-92].

1.8 Aim of this PhD thesis

Several diseases involving the immune system such as chronic-inflammatory diseases, autoimmune diseases and auto-inflammatory diseases are generated, exacerbated or maintained by the active secretion of IL-1β.

But there are several processes in which the role of IL-1β and inflammasome is not known. The aim of this thesis consists in mainly three projects:

1) To investigate the possible role of inflammasome and IL-1β in acnes vulgaris;

2) To determine the role of inflammasome in sensitization/tolerization processes involved in Contact Hypersensitivity;

3) To address the mechanisms regulating inflammasome activation. Here we study in detail the role of MAPK in IL-1β processing and secretion.

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Chapter 2

Materials and Methods

2.1 General Remarks

Standard methods are briefly described in corresponding chapters.

2.2 Materials

2.2.1 Chemicals and other consumables

(2R,4R)-4-Aminopyrrolidine-2,4-dicarboxylic acid (APDC)	Alexis, Lausen, Switzerland
5'Aza-2'-deoxycytydine	Sigma, Buchs, Switzerland
Acetic acid	Fluka Chemie, Buchs, Switzerland
Acetone	Merck, Darmstadt, Germany
Acrylamide/bisacrylamide	Rot, Karlsruhe, Germany
Adenosine triphosphate (ATP)	Sigma, Buchs, Switzerland
Agarose	Sigma, Buchs, Switzerland
Alum	Alpha Caesar, Lausanne,Switzerland
Ammonium Persulfate (APS)	Sigma, Buchs, Switzerland
Ampicilin	Sigma, Buchs, Switzerland
Anisomycin	Sigma, Buchs, Switzerland
Antimycin	Sigma, Buchs, Switzerland
Bovine Serum Albumin (BSA)	Sigma, Buchs, Switzerland
Bromophenol Blue	Sigma, Buchs, Switzerland
Calcium Chloride (CaCl2)	Sigma, Buchs, Switzerland
Cell strainers 40 and 70um	BD, Allschwil, Switzerland
Complete proteinase inhibitor	Roche, Rotkreutz, Switzerland
Deoxynucleotide triphosphates (dNTPs)	Roche, Rotkreutz, Switzerland

Dimethylsufoxide (DMSO)	Sigma, Buchs, Switzerland
Ethanol	Sigma, Buchs, Switzerland
Eosin	Sigma, Buchs, Switzerland
Ethylenediaminetetraacetic acid (EDTA)	Sigma, Buchs, Switzerland
ERK inhibitor	Calbiochem, London, UK
Flasks, dishes and cell culture plates	BD, Allschwil, Switzerland
G418	Sigma, Buchs, Switzerland
Glycerol	Sigma, Buchs, Switzerland
Glycine	Sigma, Buchs, Switzerland
H2D-CFDA ROS Detector	Sigma, Buchs, Switzerland
HEPES	Sigma, Buchs, Switzerland
Hyaluronic Acid	Sigma, Buchs, Switzerland
Hydrogen Chloride (HCl)	Sigma, Buchs, Switzerland
Hydrogen Peroxide (H2O2)	Sigma, Buchs, Switzerland
JNK inhibitor II	Calbiochem, London, UK
Kanamycin	Sigma, Buchs, Switzerland
Lipopolysaccharide (crude LPS)	Sigma, Buchs, Switzerland
Lipopolysaccharide (ultrapure LPS)	Invivogen, Toulouse, France
Magnesium Chloride (MgCl2)	Sigma, Buchs, Switzerland
Methanol	Sigma, Buchs, Switzerland
Microfilter Units (0.22, 0.45 um)	Millipore, Lausanne, Switzerland
Mitotempo	Invitrogen, Lausanne, Switzerland
Mitotracker green	Invitrogen, Lausanne, Switzerland
Mitotracker deep red	Invitrogen, Lausanne, Switzerland
MitoSOX	Invitrogen, Lausanne, Switzerland
Monossodium Uric acid Cristals (MSU)	Sam’s biotech, Zurich, Switzerland
Nigericin	Sigma, Buchs, Switzerland
Nitrocellulose membrane (0.22um)	AmershamGE, Glattbrugg, Switzerland
Nonidet P40	Sigma, Buchs, Switzerland
P38 inhibitor	Calbiochem, London, UK
Parafin wax	Sigma, Buchs, Switzerland
Paraformaldehyde (PFA)	Sigma, Buchs, Switzerland
Phostop – phosphatase inhibitor	Roche, Rotkreutz, Switzerland

Potassium Chloride (KCl)	Sigma, Buchs, Switzerland
Propidium iodide (PI)	Roche, Rotkreutz, Switzerland
Protein A sepharose	AmershamGE, Glattbrugg, Switzerland
Puromycin	Sigma, Buchs, Switzerland
R837 (imiquimod)	Invivogen, Toulouse, France
Rotenone	Sigma, Buchs, Switzerland
Slides Superfrost	Thermo, Wohlen, Switzerland
Silica	Alfa Cesar, Lausanne, Switzerland
Sodium Chloride (NaCl)	Sigma, Buchs, Switzerland
Sodium dodecyl sulfate (SDS)	Sigma, Buchs, Switzerland
Sodium Hydroxide (NaOH)	Sigma, Buchs, Switzerland
Tetradecanoyl-Phorbolacetate (TPA)	Sigma, Buchs, Switzerland
Tetramethylethylenediamine (TEMED)	Sigma, Buchs, Switzerland
Triton –X100	Sigma, Buchs, Switzerland
Trisma Base(TRIS)	Sigma, Buchs, Switzerland
Tween 20	Sigma, Buchs, Switzerland
Ultrafiltration units – amicon	Millipore, Lausanne, Switzerland
Whatman 3mm paper	Whatman-GE, Glattbrugg, Switzerland
Whatman paper for wet transfer	Biorad, Cressier, Switzerland
X-RAY medical film	GE, Glattbrugg, Switzerland
Yeast Extract	Sigma, Buchs, Switzerland
Z-VAD-fmk (pan-caspase inhibitor)	Alexis, Lausen, Switzerland
Zymosan A	Invivogen, Toulouse, France

2.2.2 Protein size Standards

Prestained protein molecular weight marker	Fermentas, Nunningen, Switzerland
Unstained precision plus protein standard	Bio-rad, Cressier, Switzerland

2.2.3 DNA size standards

pUC mix DNA marker	Fermentas, Nunningen, Switzerland
1kb DNA ladder	New England Biolabs, Frankfurt, Germany

2.2.4 Cell Culture Media and supplements/additives

DMEM	Gibco, Lausanne, Switzerland
Fetal Calf Serum PAA GOLD	PAA, Colbe, Germany
Glutamax	Gibco, Lausanne, Switzerland
Hanks balanced salt solution	Gibco, Lausanne, Switzerland
Keratinocytes-SFM, EGF, BPE	Gibco, Lausanne, Switzerland
OPTIMEM	Gibco, Lausanne, Switzerland
PBS	Gibco, Lausanne, Switzerland
RPMI	Gibco, Lausanne, Switzerland
Penicilyn/Streptomycin	Gibco, Lausanne, Switzerland
Pyruvate	Gibco, Lausanne, Switzerland
Trypsin	Gibco, Lausanne, Switzerland

2.2.5 Transfection reagents

Interferin	PolyPlus, Illkirch, France
Lipofectamine 2000	Invitrogen, Lausanne, Switzerland

2.2.6 KITS

BCA Protein assay	Pierce/Thermo, Wohlen, Switzerland
Bradford assay	Bio-rad, Cressier, Switzerland
Cytotox LDH assay	Promega, Dübendorf, Switzerland

ECL Plus wb detection kit	GE, Glattbrugg, Switzerland
ECL pico wb detection kit	Thermo, Wohlen, Switzerland
ECL femto wb detection kit	Thermo, Wohlen, Switzerland
ELISAs	
Human IL-1α	R&D systems, Abingdon, UK
Murine IL-1α	R&D systems, Abingdon, UK
Human IL-1β	R&D systems, Abingdon, UK
Murine IL-1β	R&D systems, Abingdon, UK
Human IL-6	R&D systems, Abingdon, UK
Murine IL-6	R&D systems, Abingdon, UK
Human IL-8	Biolegend, Luzern, Switzerland
Human IL-10	Biolegend, Luzern, Switzerland
Human IL-12	Biolegend, Luzern, Switzerland
Human TNF-α	Biolegend, Luzern, Switzerland
Endofree plasmid mini/midi/maxi	Quiagen, Dusseldorf, Germany
Gel extraction kit	Quiagen, Dusseldorf, Germany
NBT/BCIP substrate kit	Promega, Dübendorf, Switzerland
Plasmid mini/midi/maxi	Quiagen, Dusseldorf, Germany

2.2.7 Primary antibodies

14-3-3θ	9638	cell signalling
14-3-3θ	sc-59414	santa cruz
b-actin	2020	cell signalling
b-tubulin	2128	cell signalling
ASC	alx-210-905	alexis
BAD	9292	cell signalling
BAX	2772	cell signalling
BAX	sc-20067	santa cruz
BCL-XL	2764	cell signalling
BCL-XL	sc-7195	santa cruz
beclin-1	3495	cell signalling
BIM	2933	cell signalling
caspase-1	sc-622	santa cruz
cleaved caspase-3	9664	cell signalling
COXiv	4850	cell signalling
cytocrome C	4280	cell signalling

human mature 17 kDa IL-1β	D116	cell signaling
human total IL-1β	D37	cell signaling
mouse IL-1β	ilbm	R&D systems
NLRP3	ag-20b-0014	adipogen
P38α	2371	cell signalling
P38β	2339	cell signalling
P38γ	2307	cell signalling
P38δ	2308	cell signalling
phospho BAD ser112	9291	cell signalling
phospho BAD ser136	4366	cell signalling
phospho BAD ser155	9297	cell signalling
phospho BAX ser184	ab111391	abcam
phospho BCLXL ser62	ab30655	abcam
phospho BIM ser69	4585	cell signalling
phosphoJNK	9251	cell signalling
phosphoserine	ab9332	abcam
JNK	9252	cell signalling
JNK	sc-7345	santa cruz
Thioredoxin	ab86255	abcam
Txnip	k0204-3	MBL
VDAC	4661	cell signalling

2.2.8 Secondary antibodies

Appropriate HRP-conjugated secondary antibodies were used from New England Biolabs and proteins were detected using ECL reagent, pico and femto substrates (thermo scientific).

2.2.9 siRNAs and shRNAs

siRNAs and shRNAs were bought from Sigma, Buchs, Switzerland

2.2.10 Plasmids

All plasmids were bought from addGENE

2.2.11 Bacterial strains

E.coli BL21	AmershamGE, Glattbrugg, Switzerland
OneShot TOP10	Invitrogen, Lausanne, Switzerland

2.2.12 Eukaryotic cell lines

COS-1	ATCC, Manassas, US
HEK 293-T	ATCC, Manassas, US
J774.1	ATCC, Manassas, US
THP-1	ECACC, London, UK

2.2.13 Standard buffers and solutions

PBS

NaCl	140 mM	
KCl	30 mM	
Na2HPO4	6.5 mM	
KH2PO4	1.5 mM	
pH adjusted to 7.4		

TBST

NaCl	0.15 M	
Tween 20	0.05%	
TRIS/HCl	10 mM	(pH 8.0)

Co-IP buffer

NaCl	10mM	
EDTA	15 mM	
Triton-X100	0.1%	
TRIS/HCl	50 mM	(pH 7.5)

Chapter 3

Sensing of *Propionibacterium acnes* by the NLRP3-inflammasome drives inflammation in acne

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5. Co-senior authors

In this work, Samuel Gehrke contributed with designing and performing experiments and analysing results.

Abstract

Acne vulgaris is a potentially severe skin disease associated with colonization of the pilosebaceous unit by the commensal bacteria *Propionibacterium acnes* (*P. acnes*) and inflammation. *P. acnes* is considered to contribute to inflammation in acne, but the pathways involved are unclear. Here we reveal a previously unknown mechanism that regulates inflammation in acne. We show that interleukin-1β (IL-1β) mRNA and the active processed form of IL-1β are abundant in human inflammatory acne lesions, and identify *P. acnes* as a trigger of monocyte-macrophage NLRP3-inflammasome activation, IL-1β processing and secretion. In mice, cutaneous neutrophilic inflammation induced by *P. acnes* is critically dependent on IL-1β and the NLRP3-inflammasome of myeloid cells, but independent of toll-like receptor 2 (TLR2). These findings show that the commensal *P. acnes* - by activating the inflammasome - can trigger an innate immune response in the skin that contributes to inflammatory acne, thus establishing the NLRP3-inflammasome and IL-1β as critical therapeutic targets in acne.

Acne vulgaris is a chronic inflammatory disease of the pilo-sebaceous unit. It is the most common skin disease, affecting approximately 80% of individuals at some time during their lives[1]. The disease is multifactorial, and four processes are considered to play a pivotal role in the formation of acne lesions, namely: increased sebum production; altered follicular keratinization; inflammation; and bacterial colonization of the pilo-sebaceous unit by *Propionibacterium acnes* (*P. acnes*). Androgen-induced increased sebum production and follicular hyperkeratinisation/plugging are thought to be initial steps leading to a change of the pilo-sebaceous milieu that favors proliferation of *P. acnes*[2]. Pro-inflammatory cytokines including interleukin-1α (IL-1α) and tumour necrosis factor α (TNF-α) are, amongst others, considered to date to be responsible for the further follicular hyperkeratinization and the inflammatory lesions characteristic of acne[2-4]. Controversy exists however concerning the significance of the commensal *P. acnes* in the development of acne[2]. It has been suggested that *P. acnes* may contribute to the development of inflammatory lesions by releasing chemotactic substances, and by acting on TLR2 to stimulate the secretion of IL-6 and IL-8 by follicular keratinocytes, and IL-1β, TNF-α, IL-8 and IL-12 by monocytic cells[2,5-7]. Direct evidence of relevant triggers of inflammation in acne *in vivo*, and the precise molecular mechanisms involved, remain however largely unknown.

Innate immunity is our first line of defense against pathogens (pathogen-associated molecular patterns, PAMPs) and danger (danger-associated molecular patterns, DAMPs). Responses to PAMPs and DAMPs are mediated by germ-line encoded pattern recognition receptors (PRRs). Among the PRRs, certain Nod-like receptor (NLRs) family members including NLRP1, NLRP3, NLRC4 and AIM2, have the ability to mediate responses to PAMPs and DAMPs and subsequently assemble to form high-molecular weight, caspase-1-activating molecular platforms called inflammasomes, that control the activation and secretion of the proinflammatory cytokines IL-1β and IL-18[8,9]. Based on the observations that deregulation of the inflammasome and IL-1β has been linked to a rare genetic autoinflammatory syndrome associating pyogenic arthritis, pyoderma gangrenosum and acne, named PAPA syndrome[10], and furthermore, that acne is characterized by neutrophilic infiltration of the skin, a distinctive feature observed in transgenic

mice carrying a mutation in the NLRP3 gene equivalent to the human mutation associated with Muckle-Wells Syndrome[11], we considered that the inflammasome and IL-1β may be directly involved in the pathogenesis of acne.

In this work, we show that the proinflammatory cytokine IL-1β is abundant in its active form in human acne lesions, and that the production of IL-1β by *P. acnes*-exposed cells of monocytic lineage requires activation of the NLRP3-inflammasome. We also demonstrate in mice, that *P. acnes* reproduces neutrophilic skin lesions histologically resembling acne pustules in a NLRP3-inflammasome dependent manner. Moreover, we show that IL-1β plays a crucial *in situ* role for *P. acnes*-induced neutrophil infiltration of the skin, thus implicating IL-1β as a novel potential therapeutic target in acne.

RESULTS

P. acnes induces IL-1β secretion in monocytic cells

First, we assessed *in situ* IL-1β production in human acne lesions by immuno-histochemistry. The active, processed form of IL-1β was at the site of cutaneous inflammation, notably together with monocytes, histiocytes surrounding the pilosebaceous unit (**Fig. 1a** and Supplementary **Fig. 1**). Accordingly, we detected elevated levels (>50 mean fold increase) of IL-1β mRNA (**Fig. 1b**) selectively in skin biopsies of papulo-pustular acne. To test whether *P. acnes* is a pro-inflammatory trigger for IL-1β production observed in acne lesions, human monocytes were exposed to live or heat-inactivated *P. acnes in vitro.* Within 24 hours of exposure to *P. acnes,* significant pro-IL-1β synthesis and subsequent mature-IL-1β secretion could be detected by western blotting in cell lysates and cell culture supernatants, respectively (**Fig. 1c**). Moreover, exposure of monocytes to live or heat-inactivated *P.acnes* led to the secretion of IL-1β, IL-6, TNF-α and IL-8 in a multiplicity of infection (MOI)-dependent manner (**Fig. 1d-g**). Interestingly, monocytes secrete nanograms of IL-1β within 24h of *P. acnes* exposure (**Fig. 1d)**, without the requirement for pre-stimulation with ultra-pure LPS (upLPS), suggesting that *P. acnes* triggering of IL-1β production is biologically relevant since picograms of IL-1β are known to be already active *in vivo*[12].

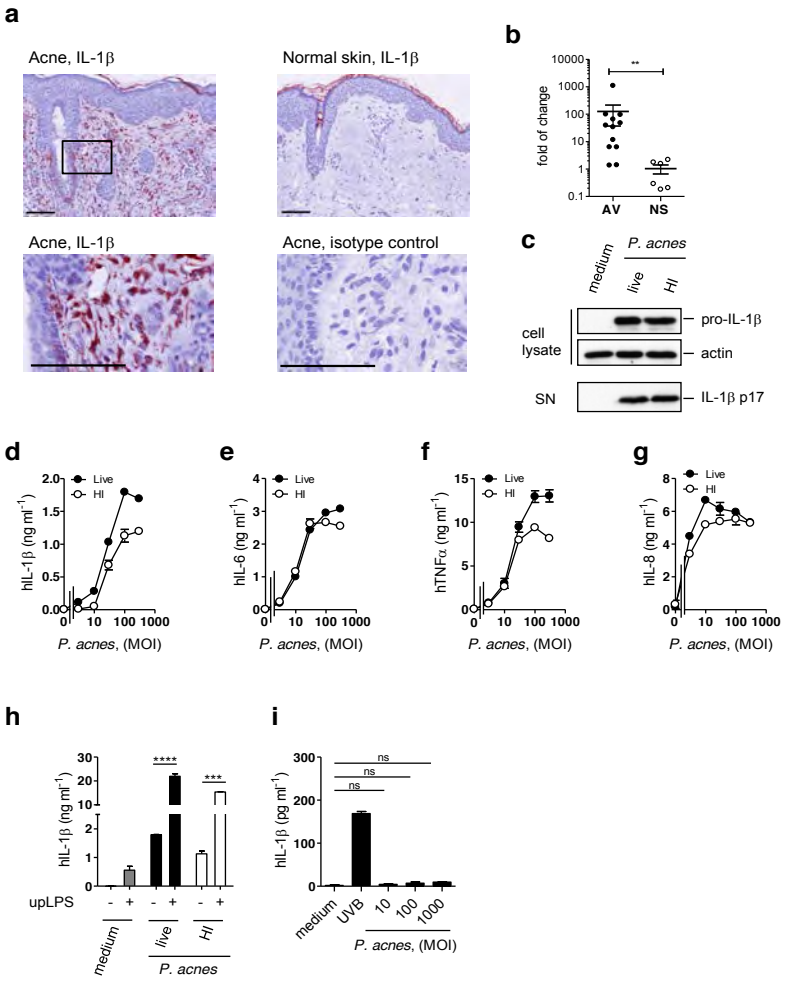

Figure 1 *P. acnes* induces cytokine secretion by myeloid cells. (**a**) Human sections of papulo-pustular acne lesions (n=10) or normal skin samples (n=10) from distinct individuals stained with anti-mature-IL-1β antibody or isotype control. Bottom left panel, high magnification of selected area from the top panel. Scale bar = 100mm. (**b**) IL-1β mRNA levels in acne lesions (AV, n=12) and normal skin (NS, n=6) measured by qPCR. The relative IL-1β mRNA abundance against ribosomal protein L27 (RPL27) mRNA levels were analyzed and normalized with the average of normal skin. **P≤0.005 by Mann–Whitney two-tailed test for acne lesions compared to normal skin. (**c**) Freshly isolated human monocytes were exposed to live or heat-inactivated (HI) *P. acnes* at multiplicity of infection =100 (MOI). Western blot analysis of pro- and mature-IL-1β p17 in cell lysates and supernatants, respectively. (**d-h**) Human monocytes were stimulated with indicated MOI of live or HI *P. acnes* for 24h. IL-1β (**d**), IL-6 (**f**), TNF-α (**g**) and IL-8 (**h**) release from *P. acnes*-exposed monocytes was determined by ELISA. (**h**) ELISA of IL1-β secreted from upLPS (100ng ml^{-1})-primed human monocytes exposed to *P. acnes*. (**i**) Primary human keratinocytes were exposed to *P. acnes* or irradiated with UVB (0.26 mW/cm^2, 6 min). After 24h, IL-1β secretion was measured by ELISA. ****$P \leq 0.00001$ and ***$P \leq 0.0001$ by one-way analysis of variance (ANOVA) with Bonferroni post-test compared to unstimulated cells (medium). Results are presented as mean and s.d and are representative of experiments performed three times.

Prior induction of pro-IL-1β synthesis (signal-1) by pre-stimulation of myeloid cells with ultra-pure LPS (upLPS) or phorbol-12-myristate-13-acetate

(PMA) resulted in stronger IL-1β secretion upon *P. acnes* exposure (**Fig. 1h** and **Supplementary Fig. 2**). Although keratinocytes are able to sense certain DAMPs such as UV and subsequently drive IL-1β-dependent innate immune responses in the skin[13-15], they were unable to secrete mature IL-1β when exposed to *P. acnes* alone or in the presence of monocyte-conditioned medium (**Fig. 1i** and **Supplementary Fig. 3**). Keratinocytes were also unable to secrete IL-6 and TNF-α upon *P. acnes* exposure, but did secrete low levels of IL-8 when exposed to very high MOI of live *P. acnes* (**Supplementary Fig. 4**).

Taken together, the above experiments suggest that cells of myeloid origin are primarily involved in the detection of *P. acnes* that leads to the mature IL-1β production occurring in inflammatory acne lesions of the skin.

P. acnes induces IL-1b secretion in a NLRP3-inflammasome-dependent manner

Enzymatic processing of the inactive precursor of IL-1β (pro-IL-1β) by caspase-1 and subsequent secretion of mature IL-1β are essential steps required for its function. Caspase-1 is initially expressed as an inactive precursor, which can be activated by the inflammasome, a cytoplasmic multiprotein complex composed of a NLRP family member, ASC and caspase-1[16]. In *P. acnes*-exposed human monocytes, the secretion of IL-1β could be inhibited in the presence of the caspase inhibitor Z-VAD (**Fig. 2a**). Moreover and in spite of normal pro-IL-1β levels (**Supplementary Fig. 5c**), THP1 cells transfected with caspase-1-shRNA (**Fig. 2b** and **Supplementary Fig. 5a**) and bone marrow-derived (BMDC) from caspase-1 deficient mice (*Casp1*$^{-/-}$) (**Fig. 2e**) were unable to secrete IL-1β either upon *P. acnes* exposure or stimulation with the inflammasome activators monosodiumurate crystals (MSU) or nigericin (**Supplementary Fig. 5d**). In contrast, monocyte production of IL-6, TNF-α and IL-8 was not inhibited by Z-VAD, demonstrating that the secretion of these cytokines upon *P. acnes* exposure is independent of inflammasome activation and IL-1β signaling (**Supplementary Fig. 6**). Furthermore, THP1 cells transduced with ASC-shRNA (**Fig. 2c** and **Supplementary Fig. 5a**) or NLRP3-shRNA (**Fig. 2d** and **Supplementary Fig. 5a**)

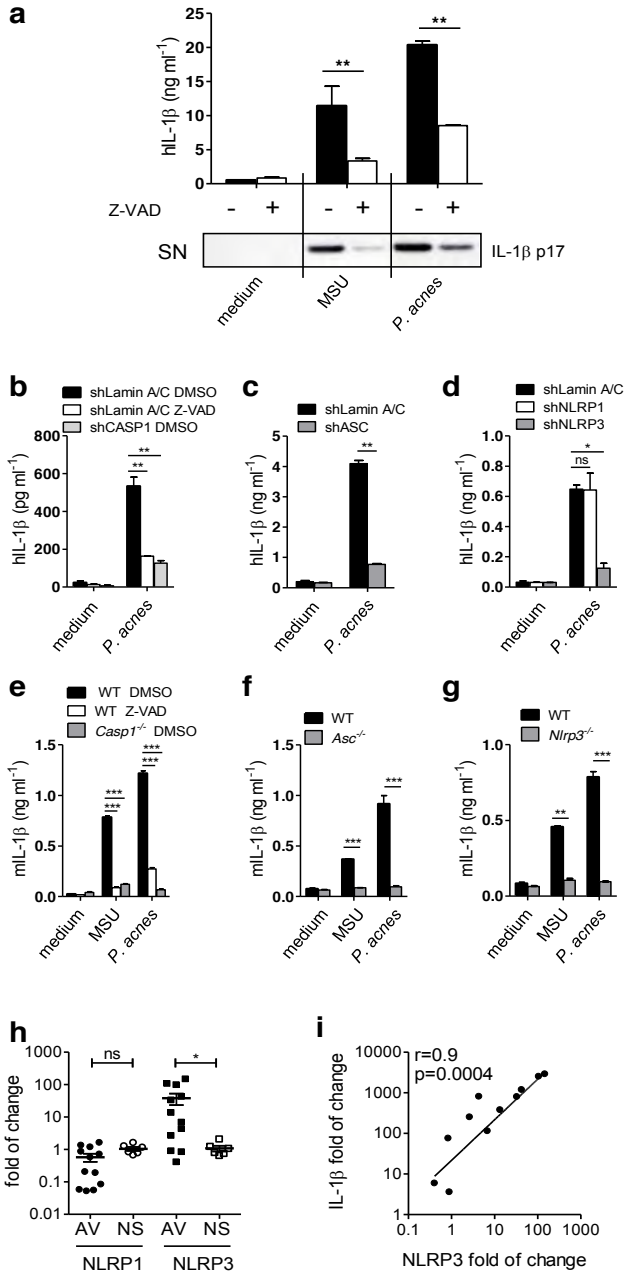

Figure 2 *P. acnes* activates caspase-1- and the NLRP3-inflammasome. (**a**) Human monocytes were primed with upLPS and exposed to medium, live *P. acnes* (MOI=100) or MSU (150mg ml^{-1}) in the presence of vehicle (DMSO) or pan-caspase inhibitor (Z-VAD-fmk, 10mM) for 24h. Supernatants were tested for IL-1β secretion by ELISA and for mature-IL-1β release (IL-1b p17) by western blot. (**b-d**) ELISA of secreted IL-1b from PMA (500 nM) -differentiated THP-1 cells transfected with shRNA to Caspase-1 (shCASP1) (**b**), ASC (shASC) (**c**), NLRP1 (shNLRP1) or NLRP3 (shNLRP3) (**d**) and exposed to live *P. acnes* (MOI=100). Lamin A/C shRNA (shLamin A/C) was used as irrelevant control. (**e-g**) ELISA of secreted IL-1β from WT, *Caspase-1*$^{-/-}$ (*Casp1*$^{-/-}$) (**e**), *Asc*$^{-/-}$ (**f**), *Nlrp3*$^{-/-}$ (**g**), murine bone-marrow-derived dendritic cells (BMDC) pulsed with upLPS and exposed to medium, *P. acnes* (MOI=300) or MSU crystals in the presence of vehicle (DMSO) or Z-VAD-fmk where indicated. (**h**) NLRP1 and NLRP3 mRNA levels in human acne lesions (AV, n=12) and normal skin (NS, n=6) measured by qPCR. The relative NLRP1 and NLRP3 mRNA abundance against RPL27 mRNA levels were analyzed and normalized with the average of normal skin. *P≤0.05 by Mann–Whitney two-tailed test for acne lesions compared to normal skin. (**i**) Scatter-plot showing the correlation (Spearman's rank correlation) between the NLRP3 and IL-1β mRNA expression in human acne biopsies. (**a-g**) * $P \leq 0.05$, ** $P \leq 0.001$ and *** $P \leq 0.0001$ by one-way analysis of variance (ANOVA) with Bonferroni post-test. Mean and s.d are presented. Results are representative of experiments repeated three times.

also exhibited reduced IL-1β secretion upon *P. acnes* exposure, while pro-IL-1β synthesis was not affected (**Supplementary Fig. 5e**). To further demonstrate the role of the inflammasome in *P. acnes* induced IL-1β -secretion, we also exposed BMDC from mice deficient for ASC (*Asc*$^{-/-}$) or NLRP3 (*Nlrp3*$^{-/-}$) to *P. acnes*. Indeed, *Asc*$^{-/-}$ and *Nlrp3*$^{-/-}$ BMDC exhibited strongly reduced IL-1β secretion when compared to wild type (WT) cells (**Fig. 2f, g**).

Distinct bacterial components may be recognized by different inflammasomes. Indeed, it has been shown that NLRP3 and NLRC4 (Ipaf)[17] are also able to sense certain bacterial components. In contrast to the NLRP3-inflammasome, the NLRC4-inflammasome does not appear to be involved in *P. acnes*-induced IL-1β production by BMDC (**Supplementary Fig. 7a**). Furthermore, using shRNA-transduced THP1 cells (**Supplementary Fig. 5b**), we observed that NLRP1 is also dispensable for IL-1β secretion upon *P. acnes* exposure (**Fig. 2d**). Interestingly, and in concordance with the above observations, expression analysis in human acne biopsies revealed increased levels of NLRP3 mRNA as compared to normal skin, whereas the mRNA expression levels of NLRP1 and NLRC4 was not altered (**Fig. 2h** and **Supplementary Fig. 7b**). Importantly, in acne skin biopsies the levels of NLRP3 mRNA correlated significantly with the levels of IL-1β mRNA (**Fig. 2i**).

These data demonstrate that *P. acnes* is a potent activator of the NLRP3-inflammasome in cells of monocytic lineage.

***P. acnes*-induced NLRP3-inflammasome activation depends on bacteria uptake, lysosomal maturation, ROS and potassium efflux**

Current models of NLRP3-inflammasome activation include the generation of reactive oxygen species (ROS)[18-20], potassium efflux[18], and lysosomal damage with release and activation of cathepsin B for stimuli requiring phagocytosis such as MSU and silica[20,21]. Exposure of THP1 cells to cytochalasin D (Cyt. D), an inhibitor of actin filament assembly, abrogated the uptake of *P. acnes* (**Fig. 3a**). Likewise, IL-1β maturation and secretion in upLPS-stimulated monocytes exposed to *P. acnes* or MSU was reduced by Cyt. D, whereas the response to the non-crystalline NLRP3-inflammasome activator nigericin was not affected (**Fig. 3b**). Interestingly, both pro-IL-1β synthesis and IL-1β release from non upLPS-stimulated monocytes exposed to *P. acnes* were impaired by Cyt. D (**Fig. 3c**). These observations demonstrate that internalization of the bacteria by antigen presenting cells (APCs) is essential for both pro-IL-1β synthesis (signal-1) and its cleavage into the active, secreted form of IL-1β (signal-2). Furthermore, specific blockade of the lysosomal cysteine protease cathepsin B, but not cathepsin D resulted in decreased IL-1β secretion by *P. acnes*- or silica-exposed monocytes (**Fig. 3d**), showing that lysosomal rupture is also required for IL-1β secretion in response to *P. acnes*. In contrast, none of the cathepsin inhibitors affected IL-1β release upon exposure to exogenous ATP.

To analyze further mechanism(s) possibly leading to NLRP3-inflammasome activation upon *P. acnes* exposure, we pre-treated human monocytes with upLPS to induce generation of pro-IL-1β and subsequently exposed them to *P. acnes* in the presence of ROS inhibitors, namely diphenyleneiodonium (DPI) or ammonium pyrrolidinecarbodithioate (PDTC). Under these conditions, IL-1β secretion by monocytes exposed to *P. acnes* or MSU was reduced by the ROS inhibitors (**Fig. 3e**). It has been previously shown that triggering of ROS in human granulocytes is caused by potassium efflux[22]. Inhibiting K^+ efflux, either by increased extracellular potassium concentration or by blockade of potassium channels with glibenclamide[23] also resulted in a significant reduction of IL-1β release by *P. acnes*- or MSU-exposed monocytes (**Fig. 3f**) whereas TNF-α secretion was unaffected (**Fig. 3g**).

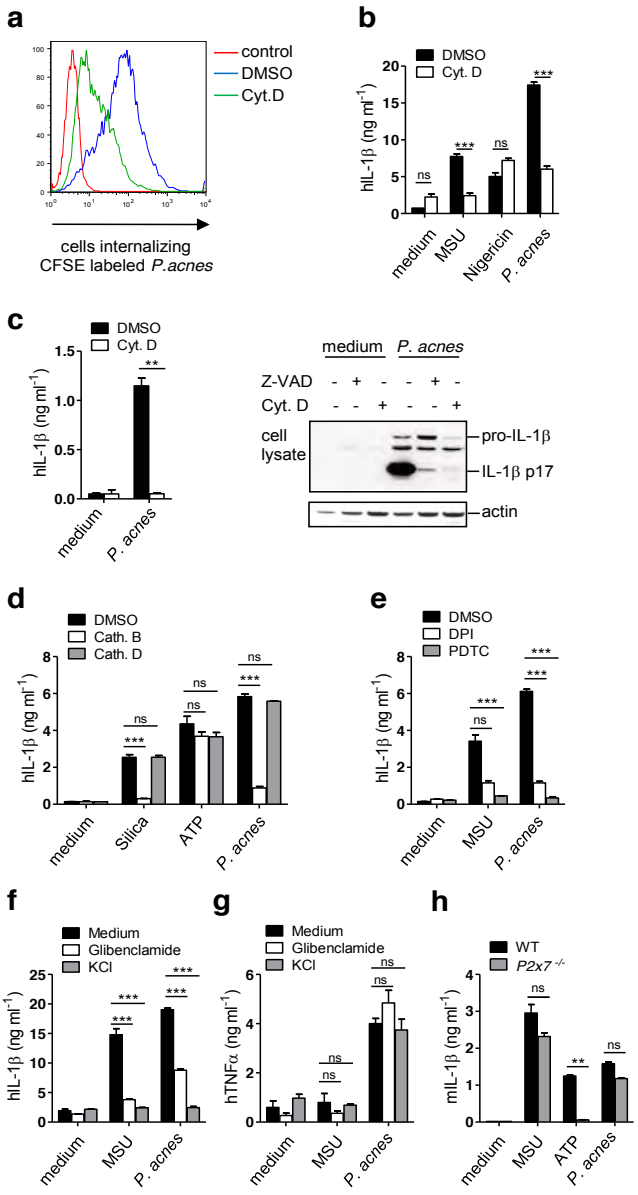

Figure 3 Inflammasome activation by *P. acnes* requires ROS production, K^+ efflux, phagocytosis lysosomal destabilization. (**a**) THP1 cells treated with vehicle (DMSO, blue line) or cytochalasin D (Cyt. D, 2.5mM, green line) were exposed to CFSE-labeled live *P. acnes* (MOI=100) for 4h or left untreated (control, red line). Bacteria uptake was analyzed by FACS. (**b**) IL-1b secretion from human monocytes primed with upLPS and incubated with vehicle (DMSO) or Cyt.D 1h prior to exposure to medium, live *P. acnes*, MSU or nigericin (20mM). (**c**) Non-upLPS-primed human monocytes were exposed for 6h to *P. acnes* in the absence (DMSO) or presence of Cyt. D or Z-VAD-fmk. ELISA of secreted IL-1β (supernatant) and western blot of pro- and mature-IL-1β p17 (cell lysates) are presented. (**d, e**) IL-1β secretion from human monocytes primed with upLPS and incubated with cathepsin B- (Cath.B, 10mM) or cathepsin D-inhibitor (Cath.D, 10mM) (**d**) or ROS inhibitors (DPI, 10mM; PDTC, 10mM) (**e**). (**f, g**) IL-1β and TNF-α release from upLPS-primed human monocytes treated with the potassium channel blockers (Glibenclamide, 100mM; KCl, 65mM) 1h prior to exposure to medium, *P. acnes* or MSU were determined after 6h by ELISA. (**h**) Secreted IL-1b from WT or *P2x7*$^{-/-}$ murine BMDC primed with upLPS and exposed for 24h to medium or *P. acnes* (MOI=300) was measured by ELISA. (**b-h**) $^{**}P \leq 0.001$ and $^{***}P \leq 0.0001$ by one-way analysis of variance (ANOVA) with Bonferroni post-test. Presented means and s.d. are representative of at least three independent experiments.

We also assessed whether cellular ATP plays a role in *P. acnes*-induced IL-1β production. BMDC from ATP receptor *P2x7*$^{-/-}$ mice released similar amounts of IL-1β as BMDC from WT mice when exposed to *P. acnes* or MSU while failing to respond to ATP (**Fig. 3h**). This suggests that *P. acnes* did not induce ATP release from exposed monocytic cells.

Taken together, these data demonstrate that inflammasome activation and the secretion of mature IL-1β by monocytes upon exposure to *P. acnes* requires phagocytosis, lysosomal damage with release and activation of cathepsin B, generation of ROS, and potassium efflux.

P. acnes induces NLRP3-inflammasome-dependent IL-1β production and cutaneous neutrophilic inflammation *in vivo*

To assess whether the NLRP3-inflammasome is involved in sensing *P. acnes in vivo*, and if IL-1β plays a role in *P. acnes*-associated cutaneous inflammation, we injected *P. acnes* intradermally in the ear of WT mice. Such intradermal injection of *P. acnes* resulted in significant ear swelling within 24 hours (**Fig. 4a**), production of pro- and mature-IL-1β (**Fig. 4b**), and a strong inflammatory response histologically comprised primarily of neutrophils (**Fig. 4c**). When inflammasome-deficient *Asc*$^{-/-}$ mice were compared to WT mice after intradermal injection of *P. acnes*, ear swelling (**Fig. 4a**), production of pro- and mature-IL-1β (**Fig. 4b**), as well as inflammation (**Fig. 4c**) were all reduced. In *Nlrp3*$^{-/-}$ mice, the magnitude of ear swelling and production of IL-1β were also reduced, similarly to *Asc*$^{-/-}$ mice (**Fig. 4a**, **b**). These observations are consistent with our *in vitro* data, and indicate that NLRP3-inflammasome-dependent IL-1β triggers neutrophilic inflammatory lesions at the site of *P. acnes* infection. To further verify this hypothesis, *P. acnes*-injected mice were treated intraperitoneally with an IL-1 receptor antagonist (IL-1Ra), a monoclonal anti-IL-1β antibody, or a TNF-α inhibitor (Etanercept) prior to intradermal administration of *P. acnes*. A significant reduction in ear swelling and neutrophilic inflammation were observed in IL-1Ra- and anti-IL-1β-treated mice as compared to TNF-α inhibitor-treated mice (**Fig. 4c, d**), thus confirming the crucial and selective role of IL-1β in the generation of cutaneous neutrophilic inflammation in response to *P. acnes* in *vivo*. In accordance with these findings, *Il-1rl*$^{-/-}$ mice also exhibited significantly

reduced ear swelling after *P. acnes* injection (**Fig. 4e**).

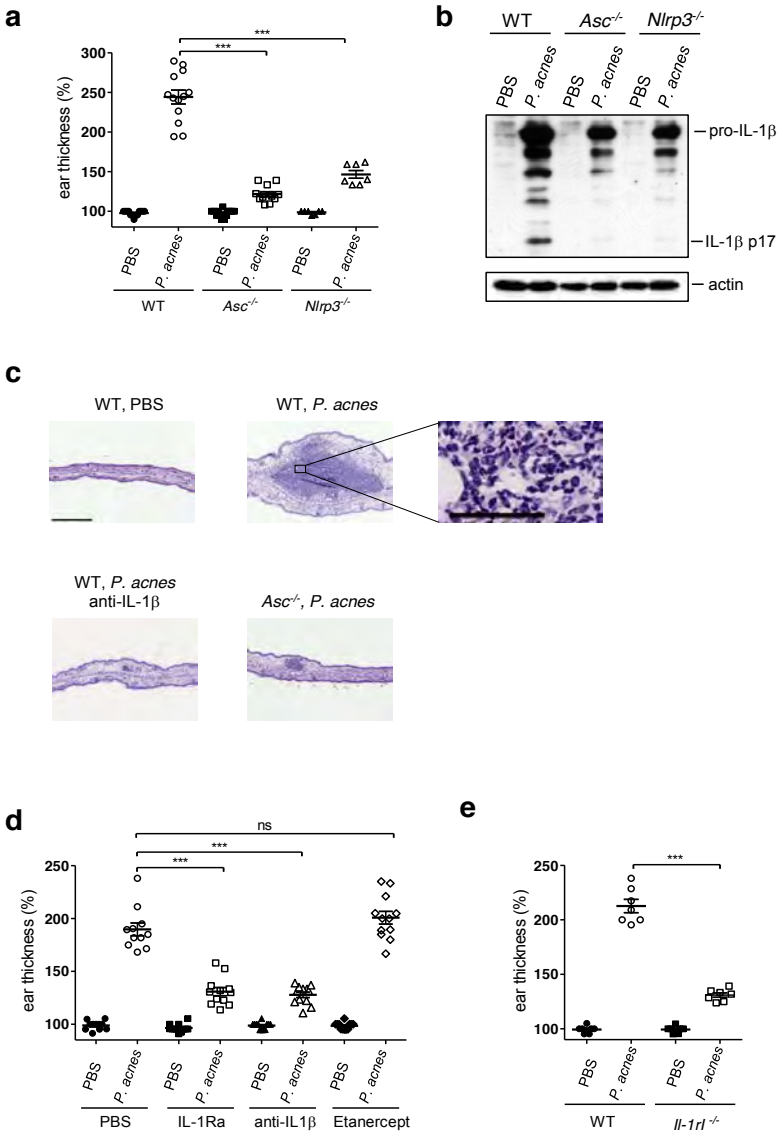

Figure 4 *P. acnes* induces NLRP3-inflammasome-dependent cutaneous inflammation *in vivo*. (**a**) WT (n=13), *Asc*$^{-/-}$ (n=13) and *Nlrp3*$^{-/-}$ (n=7) mice were injected intradermally with *P. acnes* (20x10^{6} CFU in 20ml PBS, left ear) or with an equal volume of PBS (right ear). Ear swelling as measured 24h later is presented. The ear thickness before injection was considered as 100% and served as a reference. (**b**) Pro- and mature-IL-1β western blot of ear protein extracts from WT, *Asc*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice challenged with *P. acnes*. (**c**) Histology of WT mice injected with *P. acnes*, PBS or *P. acnes* with anti-IL-1β. Histology of an *Asc*$^{-/-}$ mouse challenged with *P. acnes* is also shown. Scale bar = 500 mm. Upper right panel, high magnification of selected area from the left panel. Scale bar = 50mm. (**d**) Ear swelling in WT mice treated with vehicle (n=11), three intraperitoneal injections of IL-1Ra (Anakinra, 150mg kg^{-1}, n=13) at 8h intervals, a single i.p. injection of anti-IL-1β antibody (1mg kg^{-1}, n=12), or Etanercept (5mg kg^{-1}, n=12) 1h prior to *P. acnes* or PBS challenge. (**e**) Ear swelling in WT (n=7) and *Il-1rl* $^{-/-}$ mice (n=7) injected intradermally with *P. acnes* or with PBS as above. (**a, d, e**) *** P≤0.0001 by two tailed unpaired Student's *t* test for WT *P. acnes* challenged mice compared to mice deficient for the indicated genes or subjected to the indicated treatments.

To determine which cell types are required for triggering IL-1β-dependent inflammation upon *P. acnes* infection *in vivo*, we generated bone marrow chimeras consisting of WT mice reconstituted with either WT, *Asc*$^{-/-}$ or *Nlrp3*$^{-/-}$ bone marrow. Resulting chimeras were injected intradermally with *P. acnes* as above. Ear swelling (**Fig. 5a**) and cutaneous inflammatory infiltrates (**Fig. 5b**) were reduced in chimeric mice harboring *Asc*$^{-/-}$ or *Nlrp3*$^{-/-}$ myeloid cells when compared to mice transplanted with WT bone marrow. Moreover, the *in vivo* response to *P. acnes* was restored when *Asc*$^{-/-}$ or *Nlrp3*$^{-/-}$ mice were reconstituted with WT bone marrow (**Fig. 5c**). These data indicate that the cutaneous inflammatory response to *P. acnes in vivo* is mediated by cells of myeloid lineage. It also excludes a significant possible role of Langerhans cells, known to be radio-resistant, in the *P. acnes* induced *in vivo* inflammatory response.

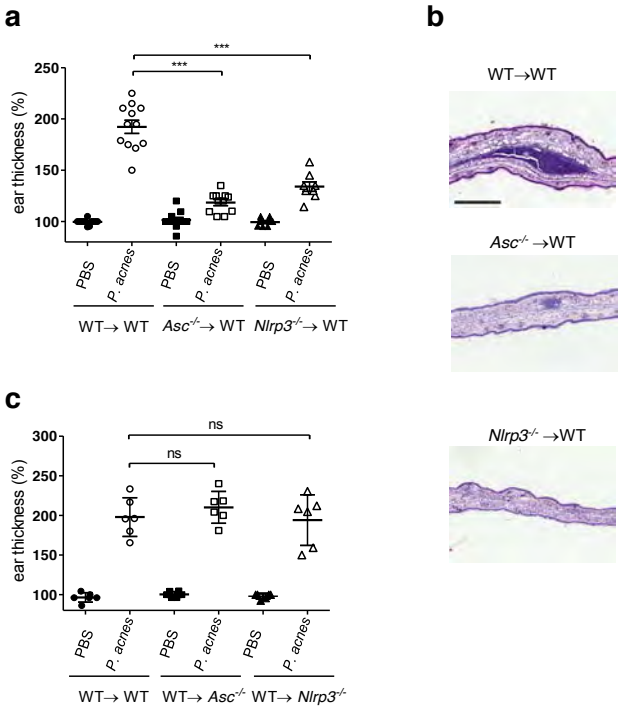

Figure 5 Cells of myeloid origin mediate immune responses to *P. acnes in vivo*. (**a, b)** WT mice reconstituted with WT (n=12), *Asc*$^{-/-}$ (n=11) or *Nlrp3*$^{-/-}$ (n=8) bone marrow were challenged with *P. acnes* or PBS as described above. The resulting swelling (**a**) and histology of the injected ear (**b**) are presented. Scale bar = 500 mm. (**c**) Ear swelling in WT (n=6), *Asc*$^{-/-}$ (n=6) or *Nlrp3*$^{-/-}$ (n=6) reconstituted with WT bone marrow and challenged *P. acnes* or PBS as above. (**a**) *** P≤0.0001 by two tailed unpaired Student's *t* test for WT reconstituted with WT bone marrow and challenged with *P. acnes* compared to mice reconstituted with bone marrow deficient for the indicated genes.

Taken together, our data demonstrate that IL-1β secreted from myeloid cells in a NLRP3-inflammasome-dependent manner, is responsible for the induction of a cutaneous neutrophilic inflammatory response to *P. acnes in vivo*.

Cutaneous inflammation induced by *P. acnes in vivo* is independent of TLR2

The pathogen recognition receptor TLR2 can trigger innate immune responses upon recognition of a broad range of PAMPs, including gram-positive bacterial factors such as peptidoglycan, lipoteichoic acid, and macrophage-activating lipopeptides[24]. It has been shown that TLR2-signaling is involved in triggering the production of proinflammatory cytokines in response to *P. acnes in vitro*[25]. We therefore assessed whether TLR2 signaling is required for the cutaneous inflammation triggered by subcutaneous *P. acnes in vivo*. After having verified that bacterial uptake was not affected by TLR2-deficiency (**Supplementary Fig. 8**), we injected *P. acnes* intradermally to *Tlr2*$^{-/-}$ mice and compared swelling and inflammatory infiltration of the injected ear to that of WT mice. In contrast to what would be predicted from previously reported *in vitro* data[25], no significant difference in ear swelling or cutaneous inflammation was observed between *Tlr2*$^{-/-}$ and WT mice (**Fig. 6a**). Production of pro-IL-1β as detected by western blotting of injected ears (**Fig. 6b**), did not require TLR2, as pro-IL-1β levels were similar in the ears of *Tlr2*$^{-/-}$ and WT mice. Furthermore, treatment of *Tlr2*$^{-/-}$ mice with IL1-Ra or anti-IL-β antibody significantly reduced ear swelling (**Fig. 6a**) and cutaneous inflammation (**Fig. 6c**), thus clearly indicating that TLR2 is dispensable for the *in vivo* cutaneous inflammatory response *to P. acnes*.

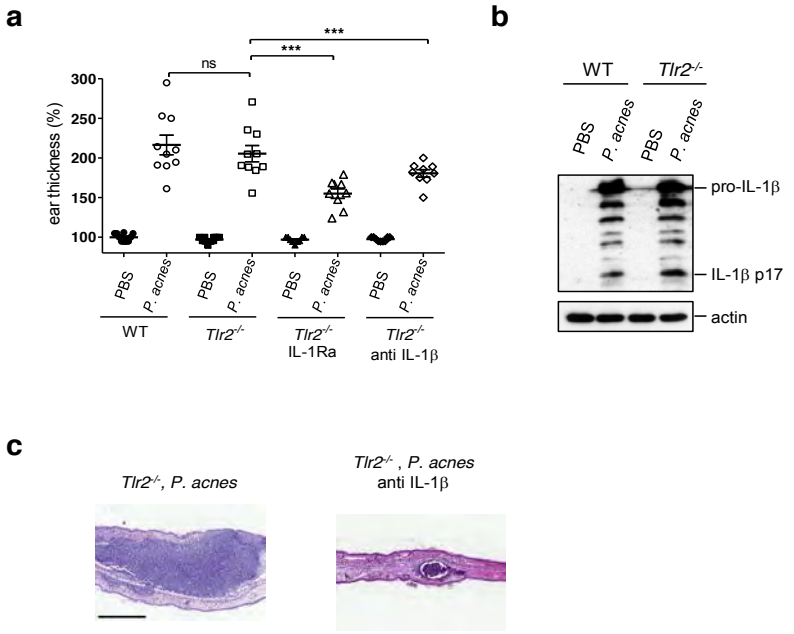

Figure 6 *P. acnes*-induced cutaneous inflammation *in vivo* is independent of TLR2. (**a-c**) WT (n=10) and *Tlr2*$^{-/-}$ mice (n=10) were injected intradermally with *P. acnes* or PBS 1h after IL-1Ra treatment (n=9) or anti-IL-1β antibody (n=9), where indicated. Ear swelling (**a**) and histology (**c**) are presented. Scale bar = 500 mm. *** P≤0.0001 by two tailed unpaired Student's *t* test for WT *P. acnes* challenged mice compared to *Tlr2*$^{-/-}$ mice for each given treatment. (**b**) Western blot analysis of pro- and mature-IL-1β in ear protein extracts from WT and *Tlr2*$^{-/-}$ mice.

DISCUSSION

Here, we show that the common anaerobic gram positive commensal of normal skin *P. acnes*, can modulate skin immune responses triggered by innate immune receptors. Indeed, sensing of *P. acnes* by myeloid cells in the skin and subsequent activation of the NLRP3-inflammasome appears to be a critical event in the formation of inflammatory skin lesions. Mature IL-1β released by myeloid cells upon sensing of *P. acnes* by the NLRP3-inflammasome likely plays a central role in driving neutrophil-rich inflammation in acne, as indicated by the high levels of IL-1β observed in human acne lesions, in mouse neutrophilic cutaneous lesions induced by *P. acnes*, and the dependence of this on the NLRP3-inflammasome. Thus we show here that

two of the four pivotal processes considered to be involved in acne lesion development - namely follicular colonization by *P. acnes* and inflammation - are linked at a molecular level, and the NLRP3-inflammasome provides this molecular link by sensing *P. acnes* and triggering IL-1β production in myeloid cells surrounding the pilosebaceous unit.

To date, the exact molecular mechanisms by which DAMPs and PAMPs lead to NLRP3-inflammasome oligomerization and activation are not fully understood. Several models have been proposed including ROS-generation, K^+ efflux and lysosomal rupture[26,27]. Here we provide evidence here that all three mechanisms are involved in *P. acnes*-mediated activation of the NLRP3-inflammasome. Noteworthy, we show that bacterial internalization due to phagocytosis by cells of myeloid origin is essential for both pro-IL-1β synthesis (signal-1) and inflammasome-dependent cleavage into its active secreted form (signal-2). It was previously unclear if and precisely how the commensal bacteria *P. acnes* can trigger an inflammatory response in the skin. Our experiments reveal that NLRP3-deficiency in myeloid cells is sufficient to prevent *P. acnes*-induced inflammation *in vivo*, and that *P. acnes* selectively induces inflammasome activation in myeloid cells but not keratinocytes *in vitro*. This suggests that triggering inflammation by *P. acnes* is dependent on location, and more specifically upon adequate access of the bacterium to myeloid cells as well as their ability to phagocytose the bacteria and trigger a NLRP3-inflammasome-dependent innate immune response. Previous studies histologically assessing early events in skin biopsies from acne patients have revealed that macrophages are present in high numbers in the immediate proximity of the pilosebaceous follicle of uninvolved acne skin as well as in early stage inflammatory lesions[28,29]. Moreover, it has been shown in skin biopsies of inflammatory acne lesions that perifollicular macrophages contain phagocytosed *P. acnes*[30]. These macrophages are likely to serve as sentinels in acne by triggering a NLRP3-inflammasome dependent inflammatory response when they gain access to *P. acnes* released into the perifollicular environment following the onset of follicular rupture provoked by follicular obstruction and intrafollicular *P. acnes* overgrowth.

Based on *in vitro* studies showing that macrophage TLR2 mediates *P. acnes*-induced cytokine production *in vitro*, it has been postulated that TLR2 could be an appropriate therapeutic target in acne[25]. Indeed, Kim *et al*. reported that the production of IL-6 by mouse peritoneal macrophages, as well as the secretion of IL-8 and IL-12 by human monocytes exposed to *P. acnes* occurs in a TLR2-dependent manner *in vitro*[25]. Although the above cytokines, notably IL-8 are known to play a role in neutrophilic inflammation, we found that *P. acnes*-induced cutaneous inflammation is TLR2-independent *in vivo.* Moreover, the production of IL-1β at the site of *P. acnes*-induced cutaneous inflammation in TLR2-deficient mice was comparable to wild-type control mice, and blockade of IL-1R and IL-1b both inhibited *P. acnes*-induced inflammation in TLR2-deficient mice. It therefore appears that, although TLR2 can sense *P. acnes* and trigger inflammatory cytokine responses *in vitro*, it may not be a critical component required for driving inflammation in response to *P. acnes* in the skin *in vivo*.

The therapy of inflammatory acne can be challenging. Lack of response, side effects, or recurrences following systemic therapy using antibiotics or retinoids are quite frequent[31]. Alternative effective therapies for acne are thus needed. Recently, clinical reports suggesting a therapeutic effect of drugs targeting IL-1β signaling in patients suffering from autoinflammatory syndromes in which acne is a symptom have appeared[32-34]. Furthermore, and in accordance with our data, recent interim results from a phase 2 double blind placebo controlled trial assessing the effect of a monoclonal antibody to IL-1β (Gevokizumab, Xoma-052) have shown that Gevokizumab given for three consecutive months induced significant improvement of inflammatory acne (http://www.clinicaltrials.gov/ct2/show/NCT01498874?term=Gevokizumab&rank=1, http://investors.xoma.com/releasedetail.cfm?ReleaseID=731711).

Taken together, we show here that *P. acnes*, one of the factors considered to contribute to acne, is a potent trigger of NLRP3-inflammasome assembly and IL-1β production in myeloid cells *in vitro* and *in vivo*. Moreover, we provide the first functional *in vivo* evidence that activation of the NLRP3-inflammasome by the anaerobic microbe *P. acnes* is a critical event in the formation of inflammatory acne lesions, and that this mechanism of

inflammation in acne can be selectively prevented by targeting inflammasome components or IL-1β. Our data thus indicates novel avenues for targeted treatment of the most debilitating features of acne, namely painful inflammatory skin lesions and subsequent scarring.

ACKNOWLEDGMENTS

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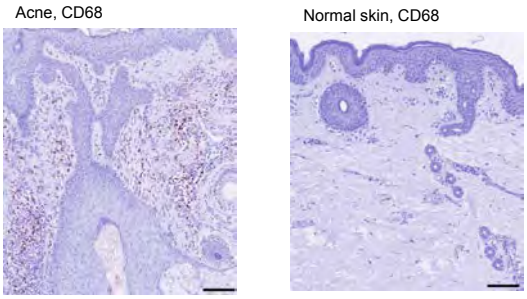
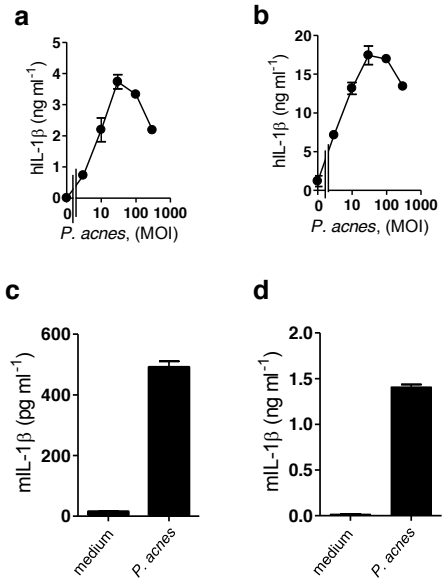
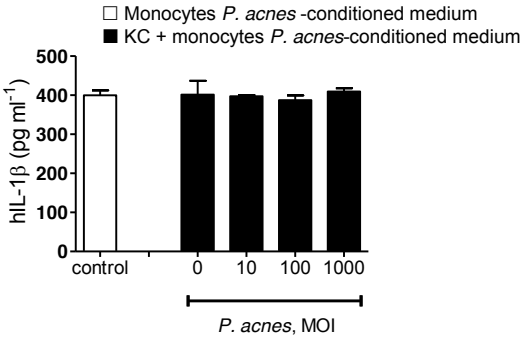
The authors declare no conflict of interest.



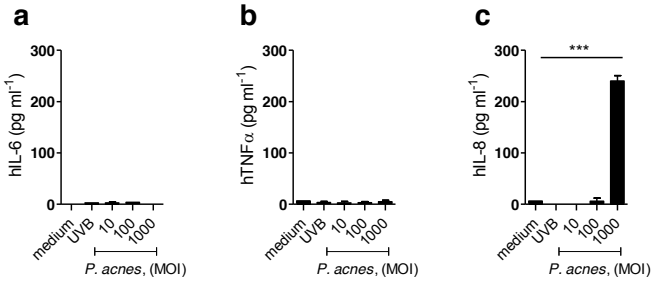
Supplementary figure 1 CD68 positive cells infiltrate acne lesions. Human sections of acne lesions (papulo-pustules, left panel) or normal skin (right panel) samples stained with anti-CD68 antibody. Scale bar = 100μm.



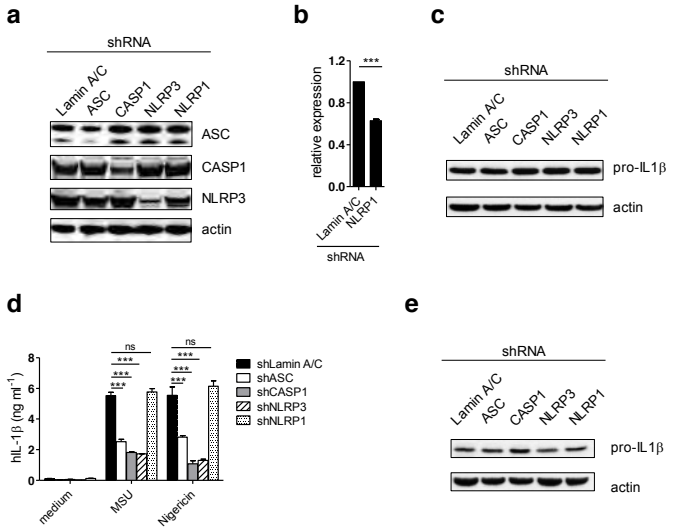
Supplementary figure 2 *P. acnes* induces IL-1β secretion in human and murine cells. Human THP1 cells untreated (**a**) or differentiated with PMA (500nM) (**b**) were exposed to indicated MOI of *P. acnes*. Murine BMDC untreated (**c**) or primed with upLPS (100ng ml^{-1}) (**d**) were stimulated with *P. acnes* (MOI=300). IL-1β release was determined by ELISA. Means and s.d. from one representative experiment of three are presented.



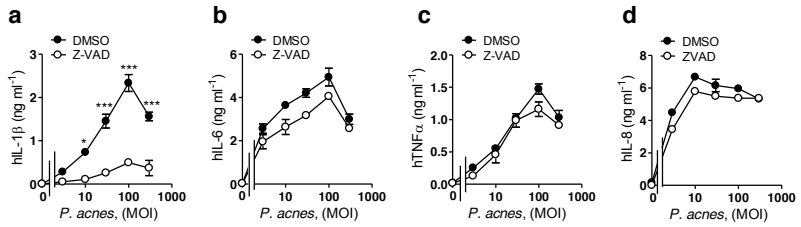
Supplementary figure 3 *P. acnes* does not induce IL-1β secretion in human primary keratinocytes. Human kerationocytes (KC) were cultured in monocyte *P. acnes*-conditioned medium (50%) and further exposed to indicated MOI of live *P.acnes* (black bars). IL-1β release was determined by ELISA. Secretion of IL-1β by infected monocytes (white bar) served as a control. Means and s.d. from one representative experiment repeated twice.



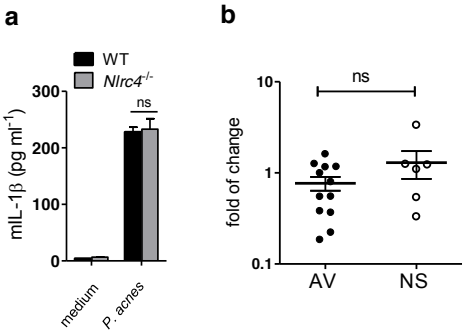
Supplementary figure 4 Cytokine secretion from human keratinocytes exposed to *P. acnes*. Primary human keratinocytes were exposed to *P. acnes* or to UVB (0.26 mW/cm^2, 6 min). After 24h, IL-6 (**a**), TNFα (**b**) and IL-8 (**c**) secretion were measured by ELISA. ***$P \leq 0.0001$ by one-way analysis of variance (ANOVA) with Bonferroni post-test compared to medium exposed cells. Results are presented as mean and s.d and are representative of experiments performed twice.



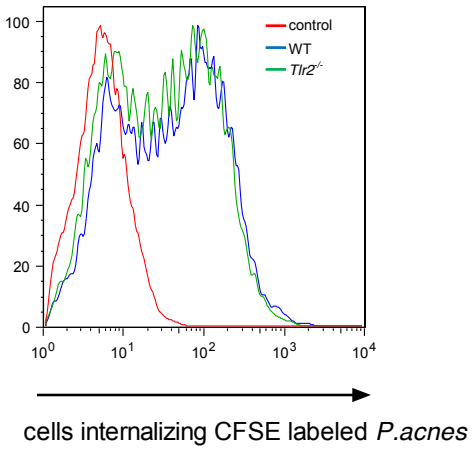
Supplementary figure 5 THP1 cells stably expressing ASC, Caspase-1 or NLRP3 gene-specific shRNA show impaired IL-1β processing. (**a, b**) THP1 cells were infected with lentivirus carrying gene-specific shRNA as indicated. The efficacy of ASC, Caspase-1 and NLRP3 knock-down was determined by western blot (**a**) and the efficacy of NLRP1 knock-down was estimated by real-time PCR (**b**). *** P≤0.0001 by two tailed unpaired Student's *t* test for THP1 NLRP1 shRNA compared to THP1 Lamin A/C shRNA. (**c**, **e**) Pro-IL-1β synthesis by THP1 cells expressing indicated shRNA upon PMA (500nM) treatment (**c**) or *P*. *acnes* infection (MOI=100) (**e**) was determined by western blot. Actin served as loading control. (**d**) The functional efficiency of each knockdown was determined by stimulation of PMA–differentiated, shRNA-transduced THP1 cells with MSU (150μg ml^{-1}) and nigericin (20μM). IL-1β release was measured by ELISA. ***$P \leq 0.0001$ by one-way analysis of variance (ANOVA) with Bonferroni post-test.



Supplementary figure 6 *P. acnes* infected monocytes secrete IL-6, TNFα and IL-8 in a caspase-1-independent manner. Freshly isolated human monocytes were stimulated with indicated MOI of *P. acnes* in the presence of vehicle (DMSO) or pan-caspase inhibitor (Z-VAD-fmk, 10μM) for 24h. Release of IL-1β (**a**), IL-6 (**b**), TNFα (**c**) and IL-8 (**d**) were determined by ELISA. $*P \leq 0.05$ and $***P \leq 0.0001$ by one-way analysis of variance (ANOVA) with Bonferroni post-test. Means and s.d. from one representative experiment of two are presented.



Supplementary figure 7 NLRC4 inflammasome is not activated by *P. acnes*. (**a**) ELISA of secreted IL-1β from *Nlrc4*$^{-/-}$ murine bone-marrow-derived dendritic cells (BMDC) pulsed with upLPS and exposed to *P. acnes* (MOI=300). (**b**) NLRC4 mRNA levels in human acne lesions (AV, n=12) and normal skin (NS, n=6) measured by qPCR. The relative NLRC4 mRNA abundance against RPL27 mRNA levels were analyzed and normalized to the average of normal skin. Results are representative of experiments performed twice.



Supplementary figure 8 *P. acnes* phagocytosis is independent of TLR2. BMDC from WT (blue line) or *Tlr2*$^{-/-}$ (green line) mice were infected with CFSE-labeled *P. acnes* (MOI=300) for 18h or left untreated (red line). Bacteria uptake was analyzed by FACS. Data are representative of one experiment repeated twice.

METHODS

Mice. *Asc*$^{-/-}$ C57BL/6 mice[35] were obtained from Genentech (San Francisco). *Nlrp3*$^{-/-}$ C57BL/6 mice[36] were obtained from Prof. Jürg Tschopp (Biochemistry Institute, University of Lausanne, Epalinges, Switzerland). *Caspase1*$^{-/-}$ C57BL/6[37] and *Nlrc4*$^{-/-}$ C57BL/6 mice[38] were kindly provided by by Wolf-Dietrich Hardt (Institute of Microbiology, ETH Zürich, Switzerland). *P2x7*$^{-/-}$ C57BL/6[39], *Il-1rl*$^{-/-}$ C57BL/6[40] were obtained from Jackson Laboratories (Bar Harbor). *Tlr2*$^{-/-}$ C57BL/6 mice [41] were kindly provided by Marc Donath (Department of Biomedicine, University Hospital Basel, Switzerland). Wild-type C57BL/6 mice were obtained from the breeding facility of the University of Zürich. All the experiments were approved by the local Animal Protection Authorities.

Human skin sampling. All human skin biopsies were collected upon approval of Local Ethical Committees and were conducted according to the Declaration of Helsinki Principals.

Bacteria. *Propionibacterium acnes* (DSM 1897, DMSZ, Braunschweig, Germany) was cultured in anaerobic conditions on cooked meat medium (Difco, Becton Dickinson) supplemented with yeast extract (5mg ml^{-1}, Sigma-Aldrich), K_2HPO_2 (5mg ml^{-1}, Fluka), resaurin (1mg ml^{-1}, Sigma-Aldrich), and cystein chloride (0.5mg ml^{-1}, Sigma-Aldrich) at 37°C. Bacteria were harvested by centrifugation at 5,000 at 10 min, washed and suspended in PBS or medium for experiments. *P. acnes* cultures were hear-inactivated by incubation at 95°C for 10 min.

Cells. Human THP1 promonocytic leukemia cells were grown in RPMI 1640 medium (Invitrogen), supplemented with 10% FCS (Invitrogen), 1% Antibiotic-Antimycotic (Invitrogen), 1 mM sodium-pyruvate (Invitrogen), 2 mM GlutaMAX solution (Invitrogen).

Monocytes from healthy donors (10 different donors) were obtained from peripheral blood mononuclear cells (PBMC). PBMC were purified from buffy-coats (obtained from Blood Donation Center in Schlieren, Switzerland) using a

density gradient (Ficoll-Paque, Pharmacia). Monocytes were sorted from PBMC using anti-CD14-labeled magnetic beads (MACS, Miltenyi Biotech, Germany) according to the manufacturer's instructions.

Mouse bone-marrow-derived dendritic cells (BMDC) were obtained by differentiation of bone marrow cells, from 6-10 weeks old mice, for 8 days in RPMI 1640 medium supplemented like above and in the presence of 20% X-63 cells (mGM-CSF-producing cells) supernatant.

Human primary keratinocytes have been cultivated as previously described[42]. Cells were irradiated with UVB (Medisun HF-54, Schulze & Böhm or UV802L, Waldmann; 0.26 mW/cm^2 for 6 min) in keratinocyte–serum-free medium (Invitrogen) or exposed to live *P. acnes*. In co-culture experiments human primary keratinocytes were stimulated with indicated MOI of live *P. acnes* in the presence of monocyte *P.acnes*-conditioned medium. Briefly, primary monocytes were stimulated with live *P. acnes* (MOI=30), conditioned medium was harvested after 24 and applied to the keratinocytes in the absence or presence of live *P. acnes*.

For infection experiments, THP1 cell were differentiated for 3h with 500 nM phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich), washed and platted one day before stimulation, human monocytes and mouse BMDC were primed overnight with ultra-pure LPS (*E. coli* 0111:B4, 100ng ml^{-1}, InvivoGen) in antibiotic-free medium. Twelve hours later, medium was replaced and cells were infected with live or heat-inactivated *P. acnes* at the indicated multiplicity of infection (MOI) or stimulated with monosodiumurate crystals (MSU, 150mg ml^{-1}), nigericin (20mM, Enzo Life Sciences), silica (500mg ml^{-1}, gift of Pr. J. Tschopp), Zymosan A (200mg ml^{-1}, Sigma-Aldrich) for indicated time. ATP (5mM, Sigma-Aldrich) that was added to the cells 30 min prior to collection of supernatants. All experiments for western blotting analysis were performed in OptiMEM serum-free medium (Invitrogen).

The following chemical inhibitors were used: Z-VAD-fmk (Z-VAD, 10mM, Enzo Life Sciences), NADPH-oxidase inhibitor dibenziodolium chloride (DPI, 10mM, Sigma-Aldrich); NO-synthase inhibitor ammonium pyrrolidinecarbodithioate (PDTC, 10mM, Sigma-Aldrich), Glybenclamide (100 mM, Sigma-Aldrich), KCl (65 mM, Fluka), cathepsin B inhibitor (CA-074Me, 10mM, Calbiochem) or cathepsin D inhibitor (Pepstatin A, 10mM, Sigma-Aldrich), cytochalasin D

(2.5mM, Fluka). Inhibitors were added 1h before cell stimulation where applicable.

Generation of THP1 cells stably expressing shRNA. THP1 cells stably expressing shRNA against lamin A/C, ASC, caspase-1, NLRP1 and NLRP3 were obtained by transducing THP1 cells with lentiviral particles. Briefly gene specific shRNA were generated by inserting oligonucleotides targeting lamin A/C, ASC, caspase-1, NLRP3[18] or NLRP1[13] into pSUPER vector (Oligoengine) and subsequent cloning into lentiviral vector pAB286.1 as described elsewhere[18]. Second-generation packaging plasmids pMD2-VSVG and pCMV-R8.91 (gift of Pr. J. Tschopp) were used for lentivirus production and infection.

Western blotting. Cell culture supernatants were precipitated by addition of 0.25 volumes of trichloroacetic acid (100% w/v, Sigma-Aldrich), 10 min incubation on ice and centrifugation for 5 min at 14,000g. Protein pellet was washed twice with ice-cold acetone (Sigma-Aldrich), centrifuged as above and dried at 70°C. Cells were lysed with buffer (10mM Tris pH 7.5, 1% NP-40, 150mM NaCl, 5mM EDTA,) containing and protease inhibitor cocktail (Roche). Proteins were separated on a NuPAGE gel (Invitrogen) in accordance with the manufacturer's protocol and transferred to a Hybond-C-Extra membrane (Amersham Biosciences) by electroblotting. The membranes were blocked with 5% gelatin (Top Block, Juro) in 1× PBS and 0.5% Tween-20 and then probed with primary antibodies as follows: rabbit polyclonal anti-human mature (17 kDa) IL-1β (D116, Cell Signaling), rabbit polyclonal anti-human IL-1β (Cell Signaling), goat polyclonal anti-mouse IL-1β (R&D Systems), rabbit polyclonal anti-ASC (AL117, Enzo Life Sciences), rabbit polyclonal anti-human caspase-1 (sc622, Santa Cruz), mouse IgG2b anti-NLRP3 (Cryo-2, Enzo Life Sciences), rabbit polyclonal anti-b-actin (Cell Signaling).
Appropriate HRP-conjugated secondary antibodies were used and proteins were detected using ECL reagent (GE Healthcare).

RNA isolation and real-time PCR. RNA were isolated from THP1 cells using the Qiagen RNeasy kit (Qiagen) following manufacturer's instructions, and total RNA was converted into cDNA by standard reverse transcription with Superscript III reverse transcriptase (Invitrogen). Quantitative PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems). The primer sequences were obtained from http://pga.mgh.harvard.edu/primerbank/:
for human RPL27: forward 5′-ATCGCCAAGAGATCAAAGATAA -3′, reverse 5′-TCTGAAGACATCCTTATTGACG-3'.
for human NLRP1: forward 5′- ATTCCAGTTTGTGCGAATCCA-3′, reverse 5′-GTTCCTTGGGGAGTATTTCCAG-3.
for human IL-1b: forward 5′-CACGATGCACCTGTACGATCA -3′, reverse 5′-GTTGCTCCATATCCTGTCCCT-3'.
for human NLRP3: forward 5′-TCTCATGGATTGGTGAACAGC -3′, reverse 5′-GGTCCCCCAGAGAATTGTCA-3'.
for human NLRC4: forward 5′- AGGTCCCACAACTCGTCAAGCT -3′, reverse 5′- TGCTCACACGATTTCCCGCCAA-3'.

The real-time PCR included an initial denaturation at 95°C for 10 min, followed by 40 cycle of 95°C for 30 s, 55°C for 1 min, 72°C for 1 min, and one cycle of 95°C for 1 min, 55°C for 30 s, 95°C for 30 s.

ELISA. Cell culture supernatants were collected at indicated time point and analyzed for presence of cytokines by ELISA. Secreted human and mouse IL-1β and IL-6 were determined by ELISA kits from R&D Systems. Human and mouse TNF-α and IL-8 ELISA kits were purchased from Biolegend. All ELISA assays were performed according to the manufacturer's instructions.

Mouse intradermal infection model. *P. acnes* (DMSZ 1897) was intradermally injected ($20x10^6$ in 20ml of PBS) into left ears of mice. Right ears were injected with an equal volume of PBS. IL-1Ra treated mice: C57BL/6 mice received three intraperitoneal (i.p.) injections of IL-1 receptor antagonist (IL-1Ra, Anakinra, Kineret 150mg kg^{-1}, Amgen) or PBS at 8h intervals. One hour after first i.p. injection of IL-1Ra mice received intradermal

injections of *P. acnes* or PBS. TNF-α inhibitor (Etanercept, 5mg kg^{-1}, Wyeth Europa Ltd) or anti-IL-1β antibody (200 mg per mouse, kindly provided by Novartis, Basel, Switzerland) were injected i.p. 24h before intradermal injection of *P. acnes* or PBS. Bone marrow chimera: indicated mice were lethally irradiated (10 Gy) and subsequently transplanted with indicated bone marrow. After hematopoietic reconstitution (8 weeks later) mice were intradermally injected with *P. acnes* or PBS.The ear thickness was measured using a micro caliper (Mitutoyo, Japan) before and 24h after bacterial injection. The percentage of ear thickness was calculated by comparing the ear thickness before (100%) and 24h after injection.

Immunohistochemistry. Human acne skin samples or mouse ear samples were cross-sectioned and stained with H&E and with rabbit anti-human mature IL-1b (Abcam, ab53175) antibody, isotype control antibody (Abcam, ab27478), or mouse-anti human CD68 (DAKO, M0876) antibody where indicated.

***P. acnes* internalization assays.** Bacteria were washed three times with PBS and then labeled with CFSE (1mM, Invitrogen) for 20 min at 37°C and extensively washed with medium. THP1 cells or BMDC were infected with indicated MOI of live *P. acnes* for 6h or 24h respectively then cells were extensively washed an analyzed by flow cytometry (FacsCanto A, BD). Where indicated, Cytochalasin D (2.5mM) was added to cells 1h before bacteria.

Statistical analysis. The statistical analysis for qPCR performed on human samples was performed using Mann–Whitney test. Data obtained from *in vitro* experiments were subjected to by one-way analysis of variance (ANOVA) with Bonferroni post-test. Data obtained from *in vivo* studies were subjected to unpaired Student's *t* test. Differences were considered significant when: * $P \leq 0.05$, ** $P \leq 0.001$, *** $P \leq 0.0001$ and **** $P \leq 0.00001$. Spearman's rank correlation analysis was used to investigate the possible correlations between NALP3 and IL-1β mRNA expression levels. All statistics were performed using GraphPad Prism software.

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Chapter 4

Danger Signaling through the Inflammasome Acts as a Master Switch between Tolerance and Sensitization

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In this work, Samuel Gehrke contributed with designing and performing experiments

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Abbreviations used in this paper: NLR, NOD-like receptor; CHS, contact hyper- sensitivity; DNFB, dinitrofluorobenzene; ASC, Apoptosis-associated speck-like protein containing a CARD domain; DNCB, dinitrochlorobenzene; TNCB, trini- trochlorobenzene; IL-1RA, IL-1R antagonist; AOO, acetone olive oil; DNTB, dinitrothiocyanobenzene; Treg, regulatory T cell.

Abstract

Efficient priming of adaptive immunity depends on danger signals provided by innate immune pathways. As an example, inflammasome-mediated activation of caspase-1 and IL-1β is crucial for the development of reactive T cells targeting sensitizers like dinitrofluorobenzene (DNFB). Surprisingly, DNFB and dinitrothiocyanobenzene provide cross-reactive Ags yet drive opposing, sensitizing vs tolerizing, T cell responses. In this study, we show that, in mice, inflammasome-signaling levels can be modulated to turn dinitrothiocyanobenzene into a sensitizer and DNFB into a tolerizer, and that it correlates with the IL-6 and IL-12 secretion levels, affecting Th1, Th17, and regulatory T cell development. Hence, our data provide the first evidence that the inflammasome can define the type of adaptive immune response elicited by an Ag, and hint at new strategies to modulate T cell responses in vivo.

The prime goal of the immune system is to protect us from harm by pathogens. To fulfill this function, it relies on sequential activation of innate and adaptive immune systems. The latter is characterized by the generation of very large repertoire of cells, each one with a specific target that proliferates rapidly when encountering the right Ag, adapting efficiently to the host environment. However, it requires clues from the less specific but faster innate immune response (1). Previously regarded as a primitive mechanism for cleaning up pathogen debris, it is now recognized as a remarkably specific system that is essential for priming adaptive immunity. Pattern recognition receptors are key sensors of the innate immune system, which are capable of recognizing molecules that are unlikely to vary much, due to their essential function for the pathogen survival. Both the transmembrane receptors called TLRs and intracellular receptors called NOD-like receptors (NLR) belong to this family (2, 3).

Although very efficient and elaborate, this dual immune surveillance is prone to making errors, such as exaggerated responses to environmental substances leading to severe skin (contact hyper-sensitivity (CHS)) or lung (asthma) diseases. Environmental chemicals may be categorized in respect to their potency as sensitizers: whereas weak sensitizers will induce a feeble adaptive immune response in a selected number of people, potent sensitizers will induce strong CHS in 100% of humans. Although several reports have shown that local irritation is important for the development of CHS (4 –7), the underlying molecular mechanisms are still largely unknown. Because Freund's adjuvant is used to boost the adaptive immune response during vaccination and has recently been demonstrated to activate both NLRs and TLRs (8), we postulated that CHS also depends on danger signaling through similar receptors.

Others and we have recently demonstrated that CHS, due to small irritant chemicals such as dinitrofluorobenzene (DNFB), is dependent on the inflammasome (9, 10). This protein complex is composed of three proteins: 1) an NLR of the NALP family (NALP, neuronal apoptosis inhibitory protein, CIITA, HET-E, TP1 (NACHT) LRR- and pyrin domain); 2) the adaptor protein apoptosis speckled-like protein with a caspase recruitment domain (ASC);

and 3) caspase-1 (3). This complex regulates the activity of caspase-1 and, consequently, of the proinflammatory cytokines IL-1β and IL-18. We found that mice lacking NALP3, ASC or the IL-1 receptor, failed to develop CHS after exposure to certain contact sensitizers, suggesting that the inflammasome plays a key role in the priming of adaptive immunity.

DNFB belongs to a family of structurally related chemical compounds with high sensitizing properties that have been used as model haptens to study effector T cell development in vivo. Dinitrochlorobenzene (DNCB) and trinitrochlorobenzene (TNCB) belong to the same family and, like DNFB, induce a strong CHS response. Dinitrothiocyanobenzene (DNTB), by contrast, fails to induce a strong immune response in vivo, despite its high structural homology to DNFB. Interestingly, several reports suggest that DNTB may induce immunological tolerance to itself and to cross-reactive dinitrophenyl compounds, in particular to DNFB (11–14). We hypothesized that the potency of a contact sensitizer may be linked to its capacity to provide danger signals mediated by the inflammasome, rather than its antigenicity, and that the inflammasome is implicated in tolerance versus sensitization mechanisms. We therefore studied the effect of DNTB and DNFB on inflammasome signaling in vitro, as well as the effect of inflammasome modulation on sensitization and tolerance in vivo.

Results

DNFB and DNTB provide similar epitopes yet differ in their capacity to activate the inflammasome and induce contact hypersensitivity

DNFB, DNCB, TNCB, and DNTB have been used as model haptens to study contact hypersensitivity and T cell reactivity in mice (11–14). As we had previously observed that TNCB activates the inflammasome in vitro and in vivo, we decided to study the effect of DNCB, DNFB, and DNTB on this signaling pathway. We found that primary keratinocytes exposed to DNFB, DNCB, and TNCB, but not DNTB, secrete significant amounts of active IL-1β, in a dose dependent manner, while the viability of the cells was not affected at these concentrations (Fig. 1*A*). This effect was inhibited by the caspase-1

inhibitor zYVAD, strongly suggesting an inflammasome dependent mechanism. Interestingly, the active component of poison ivy, urushiol, also potently activates IL-1β secretion, as previously reported (15). Because detergents with irritant properties potentiate allergic responses (4–6) and have been reported to promote IL-1β secretion by keratinoctes (15), we included SDS in our assays and found that it could also trigger IL-1β activation. Our results hinted that DNTB might fail to trigger danger signals necessary to induce CHS in vivo. Indeed, DNFB, DNCB, and TNCB induce strong sensitization (Fig. 1*B*), while DNTB is a weak sensitizer at best (11–14). The difference in the sensitizing potency of these molecules may be due to the antigenicity of these molecules, but our results rather suggest that it is linked to their capacity to provide danger signals through the activation of the inflammasome.

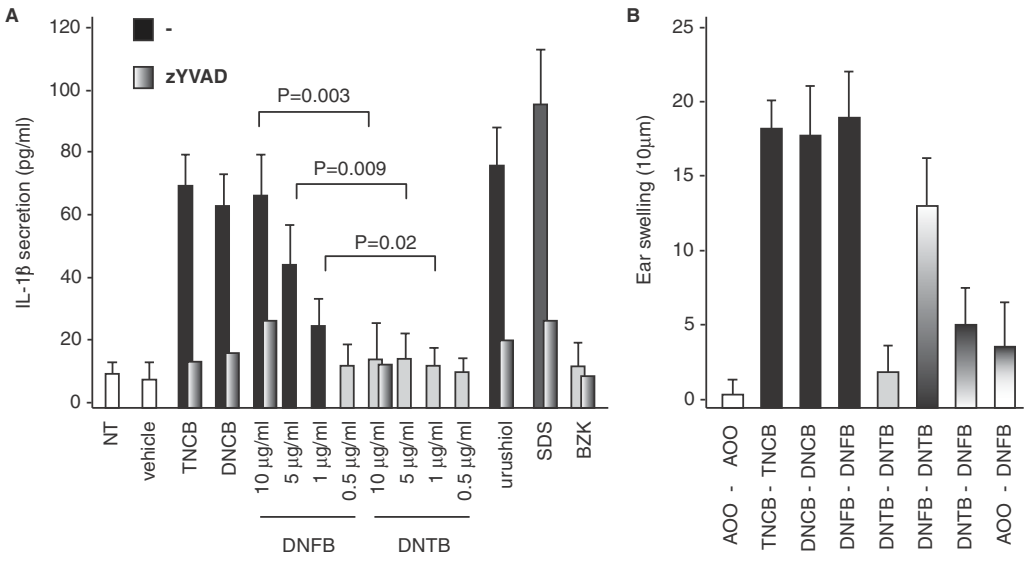

Figure 1. DNFB and DNTB provide similar epitopes, yet differ in their capacity to activate the inflammasome and induce contact hypersensitivity. *A*, Primary keratinocytes exposed in vitro to contact sensitizers such as TNCB (10 µg/ml), DNCB (10 µg/ml), DNFB (0.5–10 µg/ml), and urushiol (5 µg/ml) secrete IL-1β, whereas DNTB (0.5–10 µg/ml), which induces tolerance, does not. The skin-irritant SDS (15 µg/ml) also induced significant IL-1β release, but not benzalkonium (BZK). IL-1β activation is blocked by the specific caspase-1 inhibitor zYVAD. *B*, Mice sensitized at day 0 and 1 on the left ear, then challenged at day 5 on the right ear develop contact hypersensitivity to TNCB, DNCB, and DNFB but not to DNTB, as shown by the ear swelling 24 h after challenge. Interestingly, mice sensitized to DNFB and challenged with DNTB develop CHS, whereas DNTB-sensitized mice challenged with DNFB do not. Taken together, these findings suggest that both molecules provide cross-reactive epitope(s), but that only DNFB provides danger signals necessary to mount a CHS. (Cells: *n* = 4 independent duplicates, mice = 5–6/group).

We found that DNFB-sensitized mice display a strong reaction to DNTB (Fig. 1*B*). Our data are consistent with previously published observations and tend to confirm that DNFB and DNTB provide epitopes that can be recognized by the same T cells, yet differ in their capacity to prime the adaptive immunity (11, 12, 16). Although the value of DNTB as a tolerogen is still debated (17, 18), this suggests that the level of inflammasome signaling induced by a given molecule may better predict its sensitizing potential than the antigenic properties itself.

Danger signaling mediated by the inflammasome allows sensitization to DNTB, while blocking danger signaling prevents DNFB sensitization

IL-1β had been previously reported to play a key role for the CHS response to small irritants like DNFB, acting as an adjuvant promoting effector T cell response (19). We reasoned that whether the inflammasome is responsible for the activation of IL-1β, it should be possible to turn weak sensitizers into strong sensitizer by providing danger signals. Mice exposed twice on the right ear to DNTB failed to mount an immune response when challenged with DNFB on the contralateral ear (Fig. 2*A*), although a direct irritant effect of DNFB could be readily observed. However, when SDS was applied topically during DNTB sensitization, DNFB elicited a strong immune reaction (Fig. 2*A*). Danger signaling by these two adjuvants failed to trigger CHS in the absence of hapten co-presentation (AOO), ruling out a nonspecific effect on T cell development during the challenge phase. It did not either further enhance the response to DNFB, which can activate the inflammasome very efficiently on its own (Fig. 2*A*). These data are in accord with previous reports showing an adjuvant effect of a similar detergent (sodium lauryl sulfate) in men (4, 5). Moreover, concomitant injection of IL-1β, but not PBS alone, favored the development of CHS to DNTB (Fig. 2*A*). Conversely, the sensitizing effect of DNFB was abrogated when inflammasome signaling was inhibited, either by the local application of a caspase-1- inhibitor (zYVAD), as previously reported (20, 21). Likewise, the sensitizing capacity of DNFB was abrogated by the systemic injection of an IL-1 receptor antagonist (Anakinra) (Fig. 2*B*), which is consistent with a report showing local intradermal injection

of IL-1ra 6–24 h before sensitization (22). These results are concordant with the response of mice deficient for ASC and IL-1R.

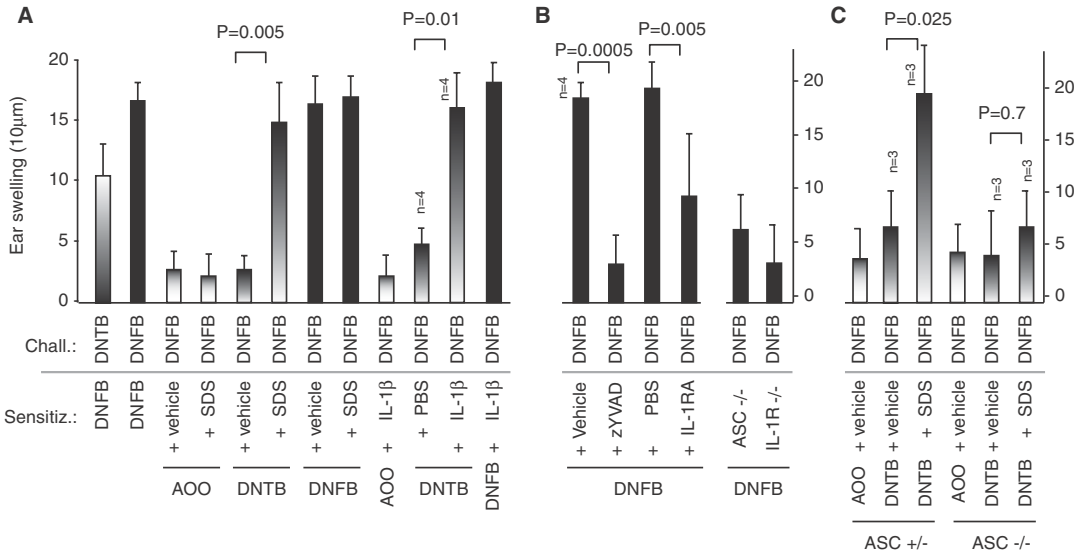

Figure 2. Concomitant danger signaling allows sensitization to DNTB, while blocking danger signaling prevents DNFB sensitization. *A*, DNTB- sensitized mice (days 0 and 1 on the left ear) challenged with DNFB (day 5 on the right ear) do not develop CHS, except if danger signals, such as i.v. IL-1β or topical SDS, are provided at the time of sensitization. Application of either SDS or IL-1β alone, in the absence of any hapten (AOO), had no effect, while neither could further increase the sensitization capacity of DNFB. *B*, Blocking of danger signals provided by DNFB during sensitization, using i.v. IL-1Ra (Anakinra) or topical zYVAD prevents the development of CHS. Similar results are obtained when the sensitization is performed in ASC or IL-1R-deficient mice. *C*, SDS increases the sensitizing capacity of DNTB in ASC$^{-/-}$ but not ASC$^{-/-}$ littermates. (*n*=5 mice/group, unless indicated in the figure). The irritant effect DNFB alone is limited, as observed in mice treated with AOO during the sensitization phase later challenged with a single application of DNFB (*A* and *C*).

Although our in vitro data strongly suggested that SDS induces IL-1β activation through an inflammasome-mediated process, we had no proof that it was the case for its in vivo adjuvant effect. We therefore assessed the effect of SDS in ASC-deficient mice. SDS potently and significantly increased the sensitizing capacity of DNTB in ASC heterozygote mice but only very weekly in their littermate ASC-deficient mice (Fig. 2*C*). Hence, the adjuvant effect we observed for SDS is mediated primarily by the inflammasome. Taken together, these data strongly suggest that the inflammasome is a key mediator of danger signals that prime the adaptive immune response directed against potent contact sensitizers.

Exposure to topical sensitizers in the absence of inflammasome-mediated danger signals leads to tolerance

The tolerogenic effect of DNTB is a matter of debate. Indeed, some find it to behave as a weak sensitizer (17, 18), whereas others find it induces tolerance (11, 13, 14). There is, on the contrary, no doubt on the fact that DNFB is a strong sensitizer. In our experimental setting, DNTB not only failed to elicit CHS, but also induced tolerance, as mice painted on two consecutive days on their shaved belly could not be sensitized any longer with DNFB, whereas the response to Oxazolone was normal (Fig. 3*A*). As DNTB and DNFB provide cross-reactive epitopes, which are different from those provided by oxazolone (16), our findings suggest that the mechanism implicated in the diminished response to DNFB is tolerance, as previously detailed (11–14) and not a more general non- specific immunosuppression.

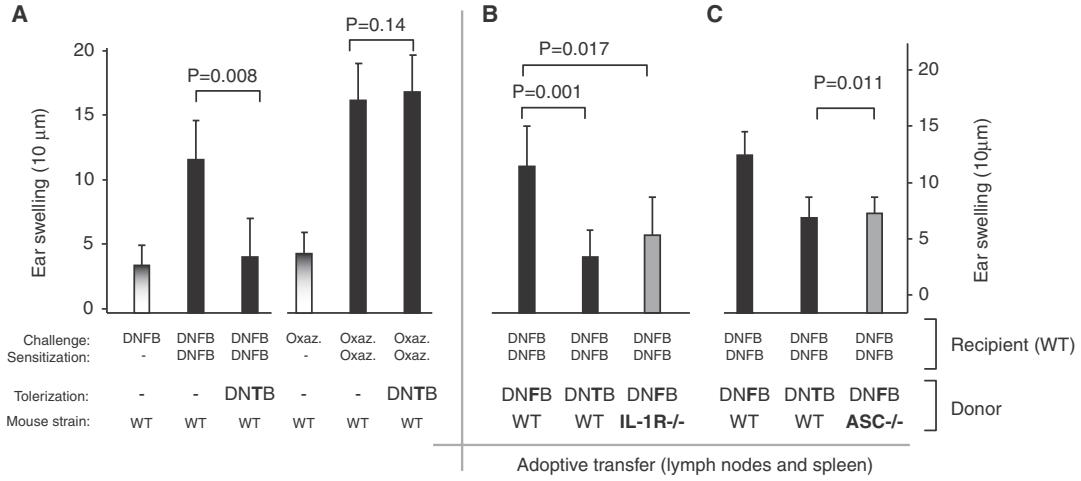

Figure 3 Exposure to topical sensitizers in the absence of inflammasome-mediated danger signals leads to tolerance. *A*, Exposure of BALB/c mice to DNTB 5 days before sensitization and challenge with DNFB results in tolerance to DNFB, as indicated by the decrease in ear swelling. Pretreatment with DNTB does not however impair the response to another contact sensitizer like oxazolone, suggesting strongly that the effect of DNTB on DNFB response is hapten specific. The irritant effect DNFB and oxazolone alone are limited, as observed in mice treated with AOO during the sensitization phase later challenged with a single application of either DNFB or oxazolone (*A*). *B*, Induction of tolerance is a cellular mediated process as adoptive transfer of cells of the spleen and lymph nodes of DNTB, but not DNFB, treated BALB/c mice blocks the development of CHS to DNFB in the recipient mice. In IL-1R deficient mice, DNFB fails to induce CHS. Adoptive transfer of cells derived from DNFB-treated mice block development of CHS to DNFB in wild-type BALB/c mice, strongly suggesting that in the absence IL-1β signaling, DNFB induces tolerance. *C*, Induction of tolerance to DNFB by DNTB is also observed in C57BL/6 mice. Adoptive transfer of cells from DNFB treated ASC-deficient mice, but not WT C57BL/6 mice, blocks CHS to DNFB in naive wild-type recipient mice, suggesting that DNFB induces tolerance in mice that cannot activate the inflammasome. (*n*= 5 mice/group).

Because we found that, in the absence of danger signaling, DNFB is no longer a potent sensitizer, we decided to test its capacity to induce tolerance in this setting. As ASC and IL-1R-deficient mice fail to mount a proper CHS to DNFB, we could not study tolerance directly in these strains (9, 10, 23, 24). However, we could expose ASC and IL-1R-deficient mice to DNFB and then transfer lymphoid cells in a wild-type recipient mouse, where sensitization and tolerance can be studied. Indeed, several groups have observed that DNTB-induced tolerance to DNFB can be transferred to a recipient syngeneic mouse, using adoptive transfer of lymph nodes and spleen cells (11, 14). We first confirmed these findings, showing that naive mice injected with lymph node and spleen extracted cells from DNTB-, but not DNFB-, treated mice failed to mount a CHS response when later sensitized and challenged with DNFB (Fig. 3*B*). The extent of tolerance induction was similar in mice directly pretreated with DNTB (Fig. 3*A*). Likewise, mice injected with cells from DNFB-primed IL-1R- or ASC- deficient mice showed a significant decrease in their capacity to mount an immune response to DNFB (Fig. 3, *B* and *C*, respec- tively), suggesting that tolerance develops in the absence of inflammasome-mediated IL-1β activation. Hence, we could demonstrate that the balance between sensitization and tolerance depends on danger signaling levels mediated by IL-1β rather than the antigenic properties of the hapten used.

Cytokine profile in tolerance vs sensitization in mouse lymph nodes

These results strongly suggested that the inflammasome determines, either at the skin or draining lymph node level, whether suppressive or effector T cells are produced. We reasoned that the cytokine environment surrounding T cells specific for the DNTB/ DNFB epitope may vary accordingly. We therefore determined the cytokine profile in draining lymph nodes of DNFB treated ears from ASC deficient mice and their control littermates. Mice sensitized with DNFB on day 1 and 2 were sacrificed at day 3 and the draining lymph nodes were dissociated and cultivated for 5 days. Under these conditions, we could not detect significant amounts of secreted IL-1β (data not shown). It is important to note here that IL-1β is secreted in

minute amounts and that IL-6, one of its bona- fide downstream target, is significantly increased after the injection of femto-moles of IL-1β, hence making IL-6 a surrogate marker for active IL1β secretion (25). Interestingly, we found that wild- type lymph nodes secreted significantly higher amounts of IL-6 and IL-12/23 than those from ASC-deficient mice, suggesting a cytokine environment likely to favor a Th1 and Th17 response and hamper regulatory T cell (Treg) development (26, 27) (Fig. 4 and see below). These results are consistent with a previous report showing that IL-12 can turn DNTB into a strong sensitizer (14). IL-10 secretion, which antagonizes LC migration and IL-12 priming of T cells (28), was slightly higher in ASC deficient mice, but the difference was not statistically significant and the very low levels observed may undermine the relevance of our finding. Hence, our results suggest but do not prove that the inflammasome may modulate the secretion levels of IL-10, which may in turn regulate T cell fate.

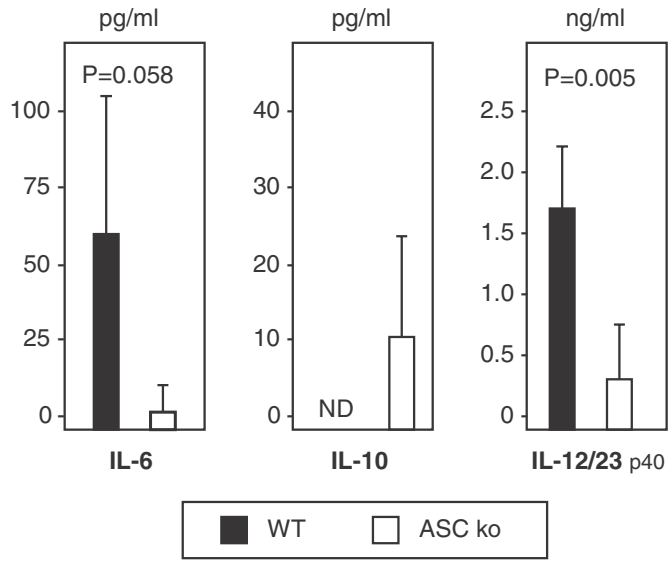

Figure 4. Cytokine profile in tolerant vs sensitized mouse lymph nodes. Lymph node cells from DNFB treated ASC$^{-/-}$ mice (days -5 and -4 before lymph nodes extraction) secrete significant amounts of IL-6 and IL-12, as determined by ELISA. In contrast, cells from their ASC$^{-/-}$ littermates secreted only small amounts of these cytokines. IL-10 levels were slightly increased in ASC$^{-/-}$ cells, yet statistical significance was not reached. (*n* =4 mice/group). ND: Non-detectable.

Mechanisms of tolerance transfer

The mechanism of DNTB induced tolerance is still controversial. However, early experiments, including adoptive transfer studies, have suggested that regulatory T cells (previously called suppressive T cells) may be implicated (11, 12, 14). We therefore studied the impact of regulatory cells on tolerance to DNFB. We first analyzed the repartition of $CD4^+$ $CD25^+$ $FoxP3^+$ T cells in lymph nodes draining the skin of DNFB treated ASC-deficient mice and their control littermates. We found that the overall number of $CD4^+$ $CD25^+$ $FoxP3^+$ cells was the same, suggesting either that Tregs are not implicated in tolerance or that the DNFB-specific Treg population variation is too small to affect the total number of regulatory T cells present in the lymph node (Fig. 5*A*), as previously reported (14). We reasoned that if Tregs do play a role in tolerance induction, adoptive transfer of Treg depleted T cells would not protect recipient mice from DNFB. We therefore extracted lymph node and spleen cells from DNTB-exposed donor mice and used magnetic bead-associated cell sorting to remove $CD4^+CD25^+$ double positive cells. We found that after adoptive transfer of these cells, sensitivity to DNFB was partially restored (Fig. 5*B*). We cannot fully rule out that this type of depletion could have a less specific general effect on Tregs numbers, affecting general repressor cytokine level, but this is unlikely because the over- all number of Treg is not affected by ASC deficiency (Fig. 5*A*). Interestingly, when DNFB-treated animals were used as donors, depletion of Treg did not induce further increase in the response to DNFB, suggesting that there are no or very few DNFB-specific Treg cells in naive mice (Fig. 5*B*). Taken together with previous reports (11, 12, 14), our results suggest that molecules which are present on the skin but do not activate the inflammasome are nevertheless presented to T cells, resulting the development of a small population of Tregs with suppressive capacity. This is consistent with the current belief that, in non-inflammatory conditions, LC travel to the lymph nodes (although at low level) and are tolerigenic (29). Moreover, this is also consistent with the observation

that the vast majority of people exposed to nickel, a weak hapten that does not activate the inflammasome (H. Watanabe, manuscript in preparation), exhibit nickel-specific Tregs with suppressive activity, but nickel-allergic patients do not (30). Hence, the Treg pathways may represent a default pathway, which is negatively regulated by danger signals, including the IL-1β activation mediated by the inflammasome.

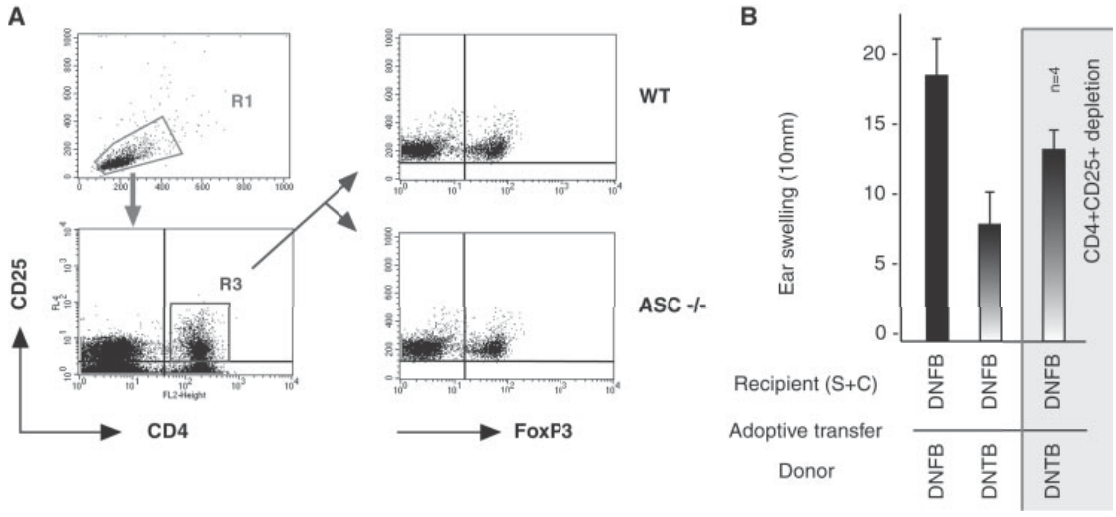

Figure 5. Mechanisms of tolerance transfer and inflammasome dependency. *A*, The repartition of $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs is the same in lymph node cells extracted from DNFB treated ASC heterozygote and deficient mice, as demonstrated by FACS staining. *B*, Adoptive transfer of DNTB exposed donor mice fails to induce tolerance to DNFB in the recipient mouse, if $CD4^+$ $CD25^+$ cells are removed by magnetic associated cell sorting, before their transfer. (*n*=4 mice/group).

Discussion

The central role of innate immunity in the activation of the adaptive immune response is no longer a matter of debate, in particular regarding TLR signaling. We herein provide evidence that danger signaling through the inflammasome can also affect the fate of the adaptive immune response. Others and we had already shown that efficient sensitization does not occur in the absence of the inflammasome and IL-1β (9, 10, 22), yet these studies did not evaluate the impact of this signaling pathway, or its absence, on the type of adaptive response elicited. We now provide evidence that the inflammasome may act as a major switch between tolerance and sensitization, laying emphasis on the sequential role of innate immunity and adaptive immunity in CHS (Fig. 6). Indeed, we found that DNTB fails to activate the inflammasome in vitro, unlike other members of this family. Furthermore, DNTB becomes a sensitizer if concomitant inflammasome signaling is provided. More importantly, blocking inflammasome signaling turns a bona fide sensitizer, DNFB, into a tolerizer. Taken together, these findings suggest that the inflammasome controls the development of tolerance or sensitization to (irritant) chemicals. Hence, NLR and TLR not only share structural resemblance, but also a similar function. Because both signaling pathways ultimately result in NF-kB activation, it is reasonable to think that a certain amount of redundancy is present. We therefore propose that stimulation of TLR, IL-1R, IL-18R, and IL-12R may all favor the development of CHS to DNTB or other Ags, as reported for IL-12 (14). Consequently, NLR activation may be an interesting target as an adjuvant for vaccines and tumor vaccination, and it should be noted here that DNCB has been used to promote inflammation and favor immune-rejection in metastatic melanoma patients (31).

Our data further suggest that the Treg-mediated development of tolerance may represent a default pathway in noninflammatory conditions, as we observed that ASC deficiency favors tolerance to haptens present in the skin. The potential physiologic benefit to this may be to avoid unnecessary/harmful T cell responses against Ags that are not accompanied by significant cell stress or damage, and therefore are unlikely to originate from a pathogen

(introducing the notion of an environmental self). This hypothesis may have important implications in the fields of virology and oncology. As an example, human papillomaviruses elicit little response from the immune system, although they can provide highly antigenic epitopes (32). Danger signaling mediated by imiquimod-induced TLR7-triggering results in a rapid destruction of the lesions by T cells (33), suggesting that the virus “invisibility” depends on the absence of inflammation. Cancer development and immune-escape may rely on similar mechanisms, as most tumors develop without eliciting inflammation. Interestingly, activation of the innate immune system through TLRs (33) or NLRs (31) may also result in an efficient antitumor response, further underlining the potential of these molecules in the field of immunotherapy (34).

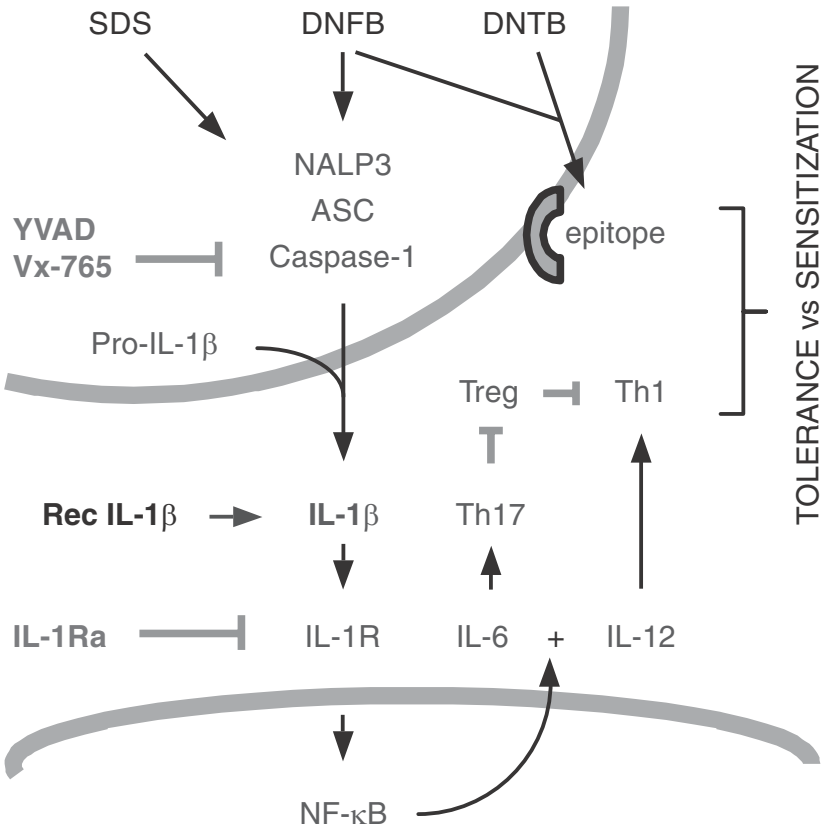

Figure 6. Putative model of the function of the inflammasome in T cell priming. DNFB-induced inflammasome activity results in the secretion of active IL-1β, which drives the expression of IL-6 and IL-12, thus creating an environment favoring Th1 development and thwarting Treg countereffects. Hence, DNFB provided at the same time a danger signal and a suitable Ag, whereas DNTB provides only an Ag. In the absence of danger signals, tolerance is the default pathway, relying on the low levels of IL-6

to stimulate Tregs. Modulation of the danger-signaling pathway can be achieved at the inflammasome level (negative: ASC deficiency, zYVAD; positive: SDS) or at the cytokine level (negative: IL-1Ra, anti-IL-12; positive: IL-1β and IL-12).

We also provide evidence that activation of the inflammasome results in the secretion of cytokines likely to favor the development of a Th1 and Th17 response counterbalancing Treg activity and tolerance. Indeed, IL-12 is known to drive T cells toward a Th1 phenotype, while IL-6 favors Th17 over Treg development (26, 27). Th17 secrete IL-17, which further decreases the activity of formerly arisen Treg cells (26, 27). Finally, decreasing IL-10 levels, a cytokine that can counterbalance the effect of IL-12 may further tip the balance in the favor of the Th1 (28). These results are in accord with a recent study showing that IL-1β breaks tolerance by favoring the expansion of $CD25^+$ effector cells (35).

Finally, our results may shed some light on the controversy regarding the sensitizing potential of DNTB. Indeed, unlike several reports, including the present work, some authors did not observe tolerance to DNTB but a weaker sensitization, concomitant with dendritic cell accumulation in regional lymph nodes (17, 18). We propose that the frontier between tolerance and sensitization is narrow and easily crossed. Indeed, in conditions where DNTB is found to induce tolerance, we found that adding danger signals turns DNTB into a sensitizer. It is likely that the nature of the vehicle, the concentration of the molecule, and skin-preparation such as shaving will tip the balance toward sensitization. In conditions where DNTB is a sensitizer, it induces similar levels of proliferation by draining lymph node cells such as DNCB, but fails to induce LC migration (36), suggesting the possible recruitment of dermal DC by DNTB (37). We propose that DNTB fails to activate the inflammasome and IL-1β activation, which is crucial for LC migration, but does activate other molecular pathways that may, depending on the context, be sufficient to trigger an efficient immune response and result in sensitization. We are currently generating LC-specific IL-1β and ASC-deficient mice to study this hypothesis. Interestingly, we have conducted experiments that indicate that the presence of an intact inflammasome is essential for DNFB and DNCB-induced LC migration (data not shown). Additional experiments are

necessary, including a characterization of the role of the inflammasome on dermal DC, as the exact role of both cell types in CHS remains controversial (38 – 41). Finally, the CHS outcome may also depend on the viral and bacterial environment of the host, as TLR and NLR activate similar targets such as NF-kB and IL-12.

Although NLR discovery is recent, several therapeutic tools targeting their signaling pathways or downstream targets are already available. For example, down-modulation of the inflammasome has been achieved successfully in patients suffering from autoinflammatory disorders, either by blocking caspase-1 or IL-1β signaling (42–44). Additional tools will soon be added to this list, including anti-IL-1b, anti-IL-6, and anti-IL12/23 Abs. As for NLR activation, Freund's adjuvant has been used extensively in vaccination studies (3, 8). We believe that gathering further insights into the role of each member of this large family will increase the number of patients benefiting from this therapeutic advance and will lead to the development of even more potent and specific drugs.

Acknowledgments

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Disclosures

The authors have no financial conflict of interest.

Materials and Methods

Cell culture and stimulation

Primary keratinocytes (human epidermal keratinocytes (Biocoba), were cultured in Epilife medium supplemented with Human Keratinocyte Growth Supplement (Biocoba) and 60 µM of Ca^{2+}. HaCaT cells were cultured in DMEM: F-12 medium (1/1) (Invitrogen) supplemented with 5 µg/ml human insulin, 10 ng/ml cholera toxin, 0.4 µg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, and 10% FCS. Cell cultures were maintained at 37°C in humidified incubators with 5% CO_2. Primary keratinocytes were pretreated with 0.1 ng/ml hTNF (Alexis) for 6 h, and stimulated with TNCB (0.5–10 µg/ml) (Fluka), DNCB (0.5–10 µg/ml), DNFB (0.5–10 µg/ml) (Fluka), DNTB (Lancaster Synthesis) (0.5–50 µg/ ml) (a gift from Ian Kimber, Faculty of Life Sciences, The University of Manchester, Manchester, U.K.), urushiol, (1–5 µg/ml) and SDS (15 µg/ ml), for 24 h with or without Z-Val-Ala-Asp-fluoromethylketone (Alexis) at 50 µM for 24 h before IL-1b secretion was determined by ELISA.

Analysis of contact hypersensitivity and tolerance in mice

Mice were handled according to institutional and Swiss Federal Veterinary Office guidelines. IL-1R and ASC-deficient mice were obtained from M. Kopf (Molecular Biomedicine ETHZ, Zürich-Schlieren, Switzerland) and V.M. Dixit (Genentech, San Francisco). For classical CHS studies: 6–7- week-old mice were sensitized by topical (external) applications of 20 µl of 0.5% TNCB, DNCB, DNFB, oxazolone, or 1% DNTB in acetone-olive oil (AOO) or AOO alone to the skin of the right ear at days 0 and 1. Elicitation was done at day 5 by topical application of 20 µl of 0.3% DNCB, DNFB, oxazolone, or 1% DNTB in AOO to the contra-lateral ear. Ear thicknesses were measured with a digital thickness gauge (Mitsutoyo) before and 24 h after hapten challenge. For tolerance induction, mice were shaved on the belly on day -1, then treated with 20 µl of 0.5% DNTB or DNFB on day 0 and 1, then sensitized on the right ear and challenged on the left ear as described above. For adoptive transfer, mice were shaved on the belly on day -1, then treated with 20 ul of 0.5%

DNTB or DNFB on day 0 and 1, sacrificed on day 5. Single cell suspension from inguinal, lymph nodes and spleen were washed in DMEM medium, counted, concentrated, and injected in the tail vein of strain-matched naive mice. Alternatively, single cell suspension obtained from the lymph node and spleen of DNTB-treated mice were depleted of $CD4^+/CD25^+$ cells using a two-step magnetic sorting (AutoMACS and Treg depletion kit, Miltenyi Biotec) according to the manufacturer's protocol. Recipient mice were sensitized on the right ear with 20 µl of 0.5% DNFB on day 1 and 2 after adoptive transfer, then challenged on the left ear on day 6, as described above.

SDS, IL-1β, zYVAD, and IL-1R agnoist (IL-1RA) administration

Twenty microliters of either SDS in dimethylformamide (1% w/v) or dimethylformamide alone was applied 30 min before each sensitization with DNTB. Ten microliters of either recombinant mIL-1b (BD Biosciences) in PBS (200 ng/ml) or PBS alone was injected s.c. in the ear 30 min before each sensitization with DNTB. Twenty microliters of either acyloxy-Z- Val-Ala-Asp-chloromethylketone (Alexis) in DMSO (0.2 mM) or DMSO alone was applied on the right ear 30 min before sensitization with DNFB on days 0, 1, and 2. One hundred microliters of IL-1R antagonist (Amgen) (150 ug/ul) or PBS was injected i.p. twice daily for three consecutive days, starting 12 h before the first sensitization with DNFB.

Cytokine detection by ELISA

Supernatants from primary keratinocyte cultrures were analyzed by ELISA for IL-1β (BD Biosciences) according to the manufacturer's instructions. ASC or wild-type littermates were treated with 20 µl of 0.3% DNFB on day 0 and 1 were sacrificed on day 2. Single cell suspension of draining neck lymph nodes were obtained and cultivated in DMEM medium for 5 days. Supernatants were then analyzed by ELISA IL-6 (R&D Systems), IL-10 (R&D Systems), and IL-12 (mouse IL-12/IL-23 p40 allele-specific DuoSet DY499, BD Biosciences) according to the manufacturer's instructions.

Statistical analysis Groups were compared using one-tailed Student's or Aspin-Welch's *t* tests.

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Chapter 5

A novel role for the mitogen activated protein kinases (MAPK) JNK2 and P38 in NLRP3 inflammasome activation

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In this work, Samuel Gehrke contributed with conception of the project, experimental design, performed all experiments, data analysis and wrote the manuscript.

Subject terms: immunology, biochemistry, health and medicine, disease.

Key words: MAPK, inflammasome, BCL-2 family members, mitochondria, MKP-1

Abstract

The NLRP3 inflammasome initiates inflammation upon sensing pathogen- and danger-associated molecular patterns (PAMPs/DAMPs) by regulating the secretion of caspase-1 dependent cytokines including interleukin (IL)-1β. How a wide variety of structurally distinct PAMPs and DAMPs trigger NLRP3 inflammasome activation at a molecular level is largely unknown. Here we show that the mitogen activated protein kinases (MAPK) JNK2 in myeloid cells, and P38 in keratinocytes, are required for NLRP3 inflammasome activation, by linking DAMP/PAMP sensing to mitochondrial permeabilization and mitochondrial DNA (mtDNA) release, via the mobilization of Bcl-2 associated death promoter (BAD) from the cytosol to the mitochondrial membrane. Accordingly, pharmacological inhibition or genetic silencing of JNK2/P38 and/or BAD abrogates mtDNA release, inflammasome activation and IL-1β secretion in response to DAMPs and PAMPs known to trigger NLRP3 inflammasome activation. Phosphorylation of JNK2/P38 is required for NLRP3 inflammasome activation, and the latter is modulated by MAPK phosphatase 1 (MKP-1). Furthermore, mice deficient in JNK2 present reduced peritoneal IL-1β production and neutrophil infiltration in a model of NLRP3-dependent uric acid-induced peritonitis. Thus, our results identify the MAPKs JNK2 and P38 as crucial mediators of NLRP3 inflammasome activation by distinct DAMPs and PAMPs, and identify these kinases as potential targets for therapeutic modulation of inflammasome-driven auto-inflammatory diseases.

Inflammasomes are intracellular multiprotein complexes that are activated upon cellular stress or infection and trigger the caspase-1-dependent processing of pro-inflammatory cytokines such as IL-1β. Inflammasomes are involved in acquired inflammatory diseases such as gout ([1]) and is the cause of hereditary autoinflammatory diseases such as the cryopyrin-associated periodic syndromes caused by activating mutations in the inflammasome component NLRP3. Key components of the NLRP3 inflammasome are the Nod-like receptor (NLR) NLRP3, the adaptor protein ASC and caspase-1. NLRP3 inflammasome activators include the DAMPs monosodic uric acid (MSU), alum, silica and ATP as well as the PAMPs nigericin, zymosan, and alpha-toxin of *S. aureus* ([2]). Upon detection of such varied PAMPs and DAMPs, NLRP3 recruits the adaptor protein ASC and procaspase-1, which results in caspase-1 activation and processing of cytoplasmic targets including the pro-inflammatory cytokines IL-1β and IL-18 ([3]).

The mechanisms leading to NLRP3 inflammasome activation are intensely debated, and there is currently no evidence that NLRP3 binds directly to any of its known activators. The diversity of structure in potential ligands suggests a shared mechanism that integrates signals generated by PAMPs and DAMPs to a common pathway leading to NLRP3 oligomerization and inflammasome activation. To date three distinct mechanisms have been proposed to account for NLRP3 activation, namely the generation of mitochondria-derived reactive oxygen species (ROS), cellular potassium efflux, and phagolysosomal destabilization ([4]). Furthermore, recent data suggest that NLRP3 inflammasome activation is a consequence of direct binding of oxidized mitochondrial DNA to NLRP3, or binding of the phosphorylated double-stranded RNA-dependent protein kinase PKR to NLRP3 ([5,6]). A unifying mechanism linking PAMP/DAMP signaling to inflammasome activation is unknown to date.

MAPK regulate important cellular functions including proliferation, differentiation and survival by linking extracellular signals to intracellular

responses ([7]). MAPK pathways also coordinate and integrate cellular responses to PAMPs and DAMPs, and deregulation of MAPK activity is associated with immune disorders ([7,8]). We therefore sought to investigate whether MAPK pathways may be involved in, or regulate inflammasome activation.

Our first approach consisted in a pharmacological inhibition of the conventional MAPK JNK, P38 and ERK. Mouse bone marrow-derived dendritic cells (BMDCs) were pre-stimulated with ultra-pure LPS, which induces NF-kb dependent pro-IL-1β synthesis, then incubated with selective MAPK inhibitors prior to exposure to known NLRP3 inflammasome activators. A significant reduction of IL-1β secretion was obtained with the JNK inhibitor SP600125 being comparable to the one observed with the caspase inhibitor ZVAD (Fig.1a,b). The P38 and ERK inhibitors 2-(4-Chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl-1,2-dihydropyrazol-3-one and U0126, respectively, failed to show significant similar inhibition in this experimental setting (Fig.1a,b). In line with this, exposure of BMDCs to inflammasome activators led to JNK, mainly JNK2 phosphorylation, at similar or higher levels to those induced by antimycin and anisomycin, two known inducers of MAPK activation (Fig.1c).

To determine which isoform of JNK is involved in IL-1β secretion and exclude off-target effects, we specifically silenced the expression of either JNK1 or JNK2, the two JNK isoforms present in myeloid cells, using shRNA (suppl. Fig.5). JNK2 knock-down THP-1 macrophages showed reduced IL-1β secretion when stimulated with NLRP3 inflammasome activators, whereas silencing of JNK1 did not significantly affect IL-1β secretion (Fig.1d,e). Similar results were obtained when cells were stimulated with polydA:dT, an AIM2 inflammasome activator ([9]) (suppl. Fig.1), suggesting that JNK2 is involved in both NLRP3 and AIM2 inflammasome activation.

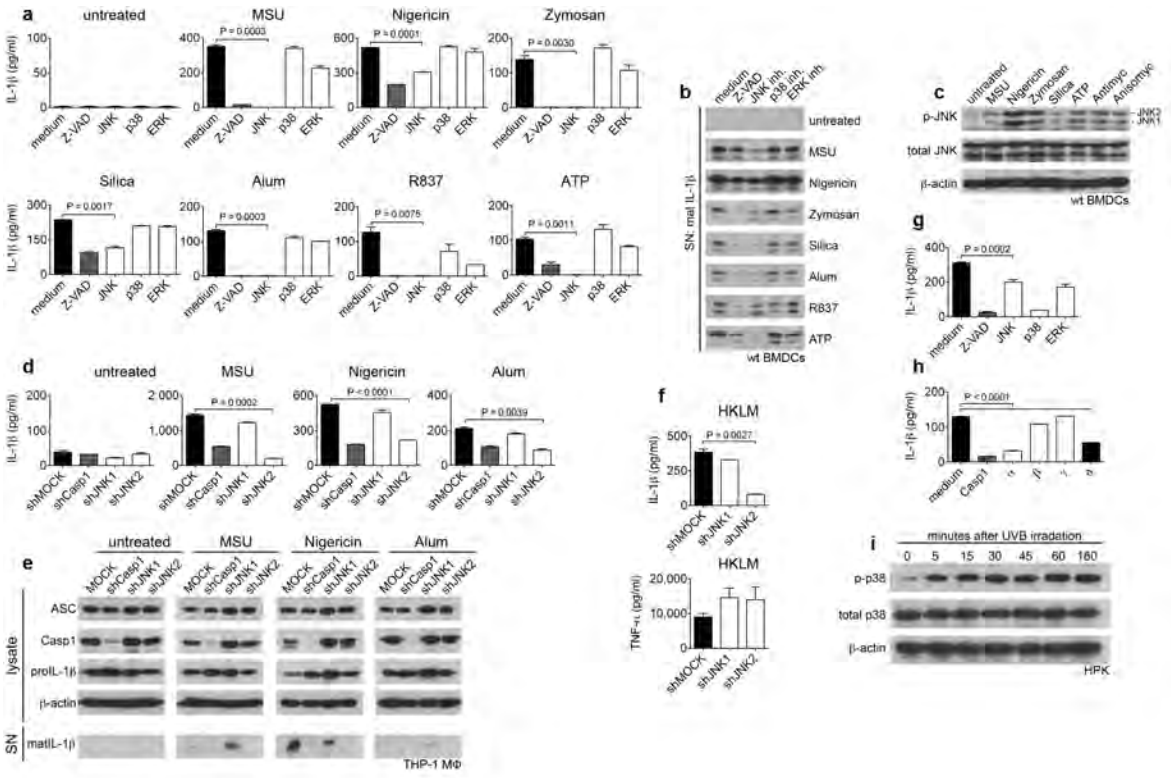

Figure.1: MAPK are involved in inflammasome activation

a,b, BMDCs were pulsed with upLPS then treated with JNK, P38 or ERK inhibitors or the caspase inhibitor ZVAD prior exposure to NLRP3 inflammasome activators. Supernatants were collected after 6h and IL-1β was analyzed by ELISA (a) and Western blot (b). **c,** BMDCs pulsed with upLPS were exposed to NLRP3 inflammasome activators and anisomycin and IL-1β was analyzed in cell lysate after 30min by Western blot as indicated. **d,e,** THP-1 macrophages stably expressing shRNA to caspase-1, JNK1 or JNK2 were differentiated with PMA or upLPS and exposed to MSU, nigericin and alum. Supernatants were collected after 6hrs and IL-1β was analyzed by ELISA (d) and Western blot (e) as cell lysates. **f,** THP-1 macrophages stably expressing shRNA to JNK1 or JNK2 were differentiated with PMA or upLPS and exposed to heat-killed listeria monocytogenes (HKLM), IL-1β and TNF-α were analyzed in supernatants after 12hrs by ELISA. **g,** human primary keratinocytes were treated with JNK, P38 or ERK inhibitor or the caspase inhibitor ZVAD prior to UVB irradiation. IL-1β was analyzed in supernatants by ELISA. **h**, human primary keratinocytes transiently expressing siRNA against caspase-1, P38α, β, γ and δ were UVB irradiated and IL-1β was analyzed in supernatants after 6hrs by ELISA. **i**, human primary keratinocytes were irradiated with UVB, and phosphoP38, total P38 and β-actin protein levels were analyzed in cell lysate by Western blot.

This effect was neither due to decreased pro-IL-1β synthesis nor to the down-regulation of inflammasome components (suppl. Fig.6, Fig.1e), nor to a general immunosuppressive effect, since TNF-α (Fig.1f) and IL-8 (suppl. Fig.4) were normally secreted despite JNK2 knock-down. In accordance with the above, peritoneal macrophages from JNK2$^{-/-}$ mice also showed reduced secretion of IL-1β (suppl. Fig.8).

To determine if MAPK signaling is also involved in the regulation of IL-1β secretion in non-myeloid cells, keratinocytes were incubated with MAPK inhibitors prior to NLRP3 inflammasome activation with UVB, or AIM2 inflammasome activation with polydA:dT. Remarkably, the P38 inhibitor 2-(4-Chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl-1,2-dihydropyrazol-3-one significantly reduced IL-1β secretion to a similar extent as the caspase inhibitor ZVAD (Fig.1g), and 2-AP a double-stranded RNA-dependent protein kinase (PKR) inhibitor (suppl. Fig.2), recently shown to be involved in inflammasome activation ([6]). Furthermore, exposure of keratinocytes to UVB resulted in a rapid increase of phosphorylated P38 (Fig.1i), and selective silencing of distinct P38 isoforms with siRNA (suppl. Fig. 7), demonstrated that the the α and δ isoforms but not the β and γ P38 isoforms are required for IL-1β secretion in UVB-irradiated (Fig.1h) and polydA:dT-transfected keratinocytes (suppl. Fig. 3).

To confirm the involvement of JNK in inflammasome activation, upLPS-pulsed BMDCs were exposed to the JNK activator anisomycin alone. This resulted in IL-1β secretion at levels similar to that induced by zymosan (Fig. 2a). In addition, overexpression of an active form of JNK2, but not JNK1, in J774.1 macrophages also resulted in spontaneous secretion of IL-1β ((Fig. 2b), whereas overexpression of a dominant negative JNK2, but not JNK1, reduced IL-1β secretion in the same cells stimulated with zymosan (Fig. 2c).

It has been recently proposed that mitochondrial loss of membrane potential and leakage of mtDNA to the cytosol are crucial events for NLRP3

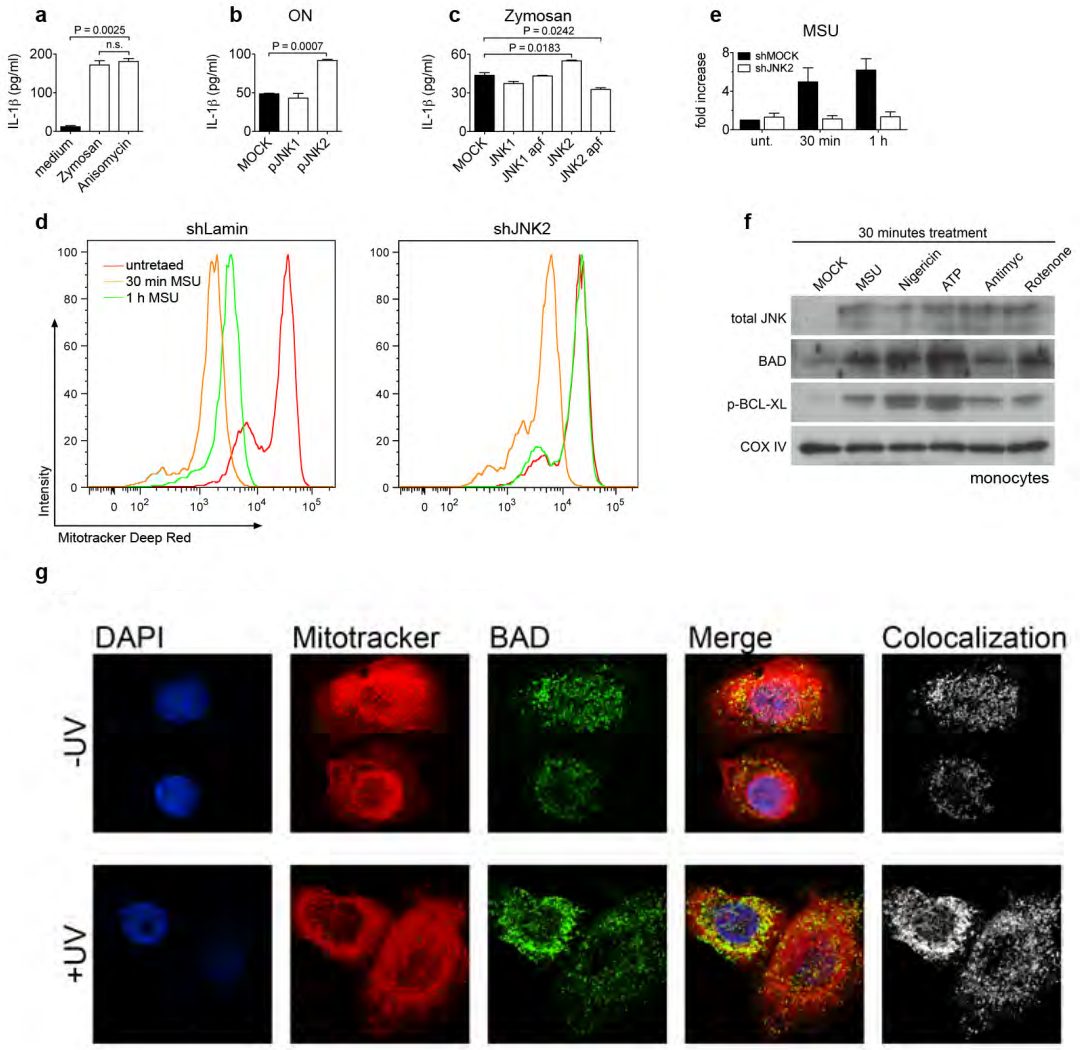

Figure 2: JNK mediates mitochondrial damage. **a,** BMDCs upLPS pulsed were exposed to zymosan and anisomycin. IL-1β was analyzed in supernatants after 6hrs by ELISA. **b**, J774.1 macrophages transiently overexpressing active JNK1 and active JNK2 were pulsed with upLPS and supernatants were collected after overnight incubation and IL-1β measured by ELISA. **c,** J774.1 macrophages transiently overexpressing JNK1, dominant mutant JNK1 (JNK1apf), JNK2 and dominant mutant JNK2 (JNK2apf) were pulsed with upLPS and exposed to zymosan. IL-1β was analyzed in supernatants by ELISA after 6hrs. **d,** THP-1 macrophages stably expressing shRNA to JNK2 were exposed to MSU for the indicated time points, and mitotracker green/deep red double positive cells are shown. **e,** THP-1 macrophages stably expressing shRNA to JNK2 were exposed to MSU for the indicated time points and mitochondrial DNA in purified cytosolic fractions were analyzed by quantitative PCR. **f,** Purified mitochondrial and cytosolic fractions from monocytes treated with the indicated inflammasome activators were analyzed by western blotting using antibodies to JNK, BAD and pBCL-XL. **g,** Human primary keratinocytes were UVB irradiated, stained with mitotracker deep red and anti-BAD and analyzed by confocal microscopy.

inflammasome activation ([5,10,11]). To assess the impact of MAPK signaling on these events, we measured mitochondrial membrane potential (ΔΨm), and analyzed the presence of mtDNA in the cytosol of THP-1 macrophages prior to, or 30min and one hour after exposure to MSU. In contrast to mock-transfected THP-1, JNK2 knock-down cells showed barely any sharp loss of ΔΨm (Fig. 2d), and virtually no cytosolic mtDNA leakage (Fig. 2e). It has been reported that JNK translocation into the mitochondria can occur upon cell stress ([12,13]). Analysis of the purified mitochondrial fraction of monocytes exposed to different NLRP3 inflammasome activators revealed significantly enhanced JNK2, but not JNK1 content in the mitochondrial fraction, suggesting that migration of JNK2 from the cytoplasma to the mitochondria is an early event in inflammasome activation (Fig. 2f).

The mechanism leading to mtDNA leakage that has been reported to be associated with NLRP3 inflammasome activation is currently unclear ([5]). Experimental evidence suggests that JNK can regulate the mitochondrial permeability transition by interacting with BCL-2 family members ([14,15]). Interestingly, BCL-2 overexpression was reported to negatively regulate NLRP3 inflammasome activation via ΔΨm modulation ([5]). To investigate if other BCL-2 members are involved in this process, we analyzed the presence of these proteins in the purified mitochondrial fraction of monocytes exposed to NLRP3 inflammasome activators. We found that NLRP3 inflammasome activation is associated with BAD translocation to the mitochondria, but not the translocation of BCL-XL (Fig. 2f, In accordance with this, using confocal microscopy, we demonstrate migration of BAD to the mitochondria after UVB irradiation in keratinocytes (Fig. 2g).

To functionally assess if BCL2 members are critical for inflammasome activation, we knocked down BAD, BCL-XL, BIM, and BAX in THP1 macrophages with shRNA. Secretion of IL-1b following MSU, nigericin and alum exposure was significantly reduced in BAD, BCL-XL and BAX but not BIM knock-down cells (Fig. 3a,b). In contrast, TNF-α and IL-8 were not affected by BAD,

BCL-XL or BAX knock-down (Ffig. 3c and suppl. Fig. 9). Similar results were observed when BAD, BCL-XL and BAX were silenced in UVB-irradiated human keratinocytes (suppl. Fig. 10), or THP-1 macrophages and keratinocytes exposed to polydA:dT (suppl. Fig. 11,12), suggesting that BAD, BCL-XL and BAX are required for NLRP3 and AIM2 inflammasome activation. Flow cytometry analysis of THP-1 macrophages exposed to MSU with mitotracker deepRed revealed a significant reduction in the loss of $\Delta\Psi m$ when BAD, BCL-XL and BAX were silenced (Fig. 3d), and the latter correlated with reduced mtDNA leakage into the cytosol (Fig. 3e). Taken together, these results provide evidence that the BCL2 family members BAD, BCL-XL and BAX are involved in the mitochondrial leakage occurring upon exposure to inflammasome activators, and this process is distinct from apoptosis, as revealed by the absence of detectable caspase-3 activity under these conditions (suppl. Fig. 13).

To further establish the involvement of BAD in inflammasome activation, we incubated upLPS-pulsed BMDCs with ABT-737, a BAD mimetic not able to induce apoptosis by itself ([16]). In the absence of exposure to PAMPs and DAMPs, ABT-737 induced slight IL-1β secretion, and this was further enhanced by combination with the JNK activator anisomycin (Fig. 3f). Thus, artificially mimicking a situation in which JNK and BAD are activated is sufficient for triggering inflammasome activation in the absence of PAMPs and DAMPs. In accordance with a key role for BAD in inflammasome activation, we could detect spontaneous secretion of IL-1β in J774.1 macrophages upon forced overexpression of BAD (Fig. 3g). Likewise, when the scaffold protein 14-3-3, also termed YWHAQ which is known to sequestrate BAD to the cytosol and prevent its mitochondrial translocation ([17]), was overexpressed, IL-1β secretion in J774.1 macrophages upon zymosan exposure was significantly reduced (Fig. 3h).

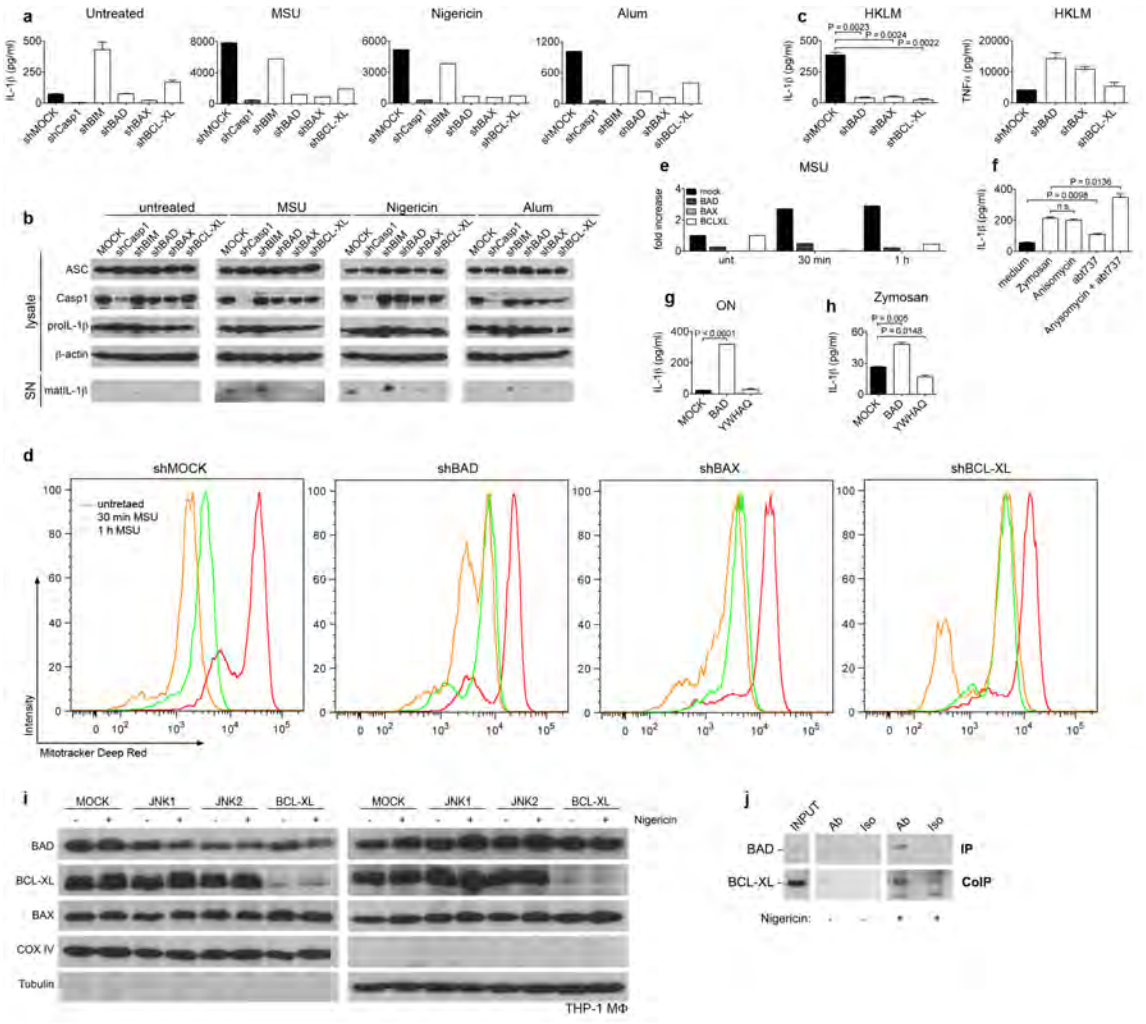

Figure 3: BCL-2 family members are involved in inflammasome activation and mitochondrial damage

a,b, THP1 macrophages stably expressing shRNA against caspase-1, BIM, BAD, BAX and BCL-XL were differentiated with PMA or upLPS and exposed to the indicated inflammasome activators. IL-1β was analyzed in supernatants by ELISA and Western blot and in cell lysates by Western blot. **c,** THP1 macrophages stably expressing shRNA to BAD, BAX and BCL-XL were differentiated with PMA or upLPS and exposed to heat killed listeria monocytogenes (HKLM). IL-1β and TNF-α were analyzed in supernatants 12hrs after exposure by ELISA**. d,** THP1 macrophages stably expressing shRNA against BAD, BAX and BCL-XL were exposed to MSU for the indicated time points. Mitotracker green/deep red double positive cells are shown. **e,** THP-1 macrophages stably expressing shRNA to BAD, BAX and BCL-XL were exposed to MSU for the indicated time points and mitochondrial DNA from purified cytosolic fractions was analyzed by quantitative PCR. **f,** upLPS pulsed BMDCs were incubated with anisomycin with or without the BAD mimetic abt737. IL-1β was analyzed in supernatants after 6hrs. **g,** J774.1 cells transiently overexpressing BAD or YWHAQ were pulsed with upLPS and IL-1β was analyzed in supernatants by ELISA after overnight incubation. **h**, J774.1 macrophages transiently overexpressing BAD or YWHAQ were pulsed with upLPS

and exposed to zymosan. IL-1β was measured in supernatants after 6hrs. **i**, Purified mitochondrial and cytosolic fractions from THP-1 macrophages stably expressing the indicated shRNA and exposed to nigericin were analyzed by Western blot. **j,** Purified mitochondrial fractions from monocytes exposed to nigericin were co-immuno-precipitated with antibodies to BAD and BCL-XL and analyzed by Western blot.

Given the evidence that JNK2 phosphorylation in myeloid cells, or P38 phosphorylation in keratinocytes, as well as BAD translocation to the mitochondria in both cell types are prerequisites for inflammasome activation, we subsequently investigated the possible link between JNK activation and BAD translocation. To this end, purified mitochondrial fractions of shRNA knock-down THP1 macrophages exposed to the inflammasome activator nigericin were analyzed. We demonstrated that BAD translocates to the mitochondria upon inflammasome activation, but this effect was decreased in JNK2 and BCL-XL knock-down (Fig. 3i). The association of BAD with BCL-XL in the mitochondria is an important event in the loss of ΔΨm ([18]). By immuno-precipitating BAD and BCL-XL from the purified mitochondrial fraction of monocytes after incubation with nigericin, we demonstrated that BAD and BCL-XL directly interacted upon inflammasome activation (Fig. 3j).

JNK and P38 kinases can be dephosphorylated and thus inactivated by MKP-1, also termed DUSP1. This phosphatase has been shown to suppress the production of pro-inflammatory cytokines as evidenced by increased inflammation and impaired bacterial clearance in MKP-$1^{-/-}$ mice ([19-21]). Following exposure of peritoneal macrophages from MKP-$1^{-/-}$ mice to NLRP3 inflammasome activators, significantly higher amounts of IL-1β secretion were detected (Fig. 4a), and this correlated with higher amounts of phosphorylated JNK 1 and 2. (Fig. 4b). Conversely, overexpression of MKP-1 in J774.1 macrophages exposed to the NLRP3 inflammasome activator zymosan, resulted in a significant reduction in IL-1β when compared to mock-transfected cells (Fig. 4c).

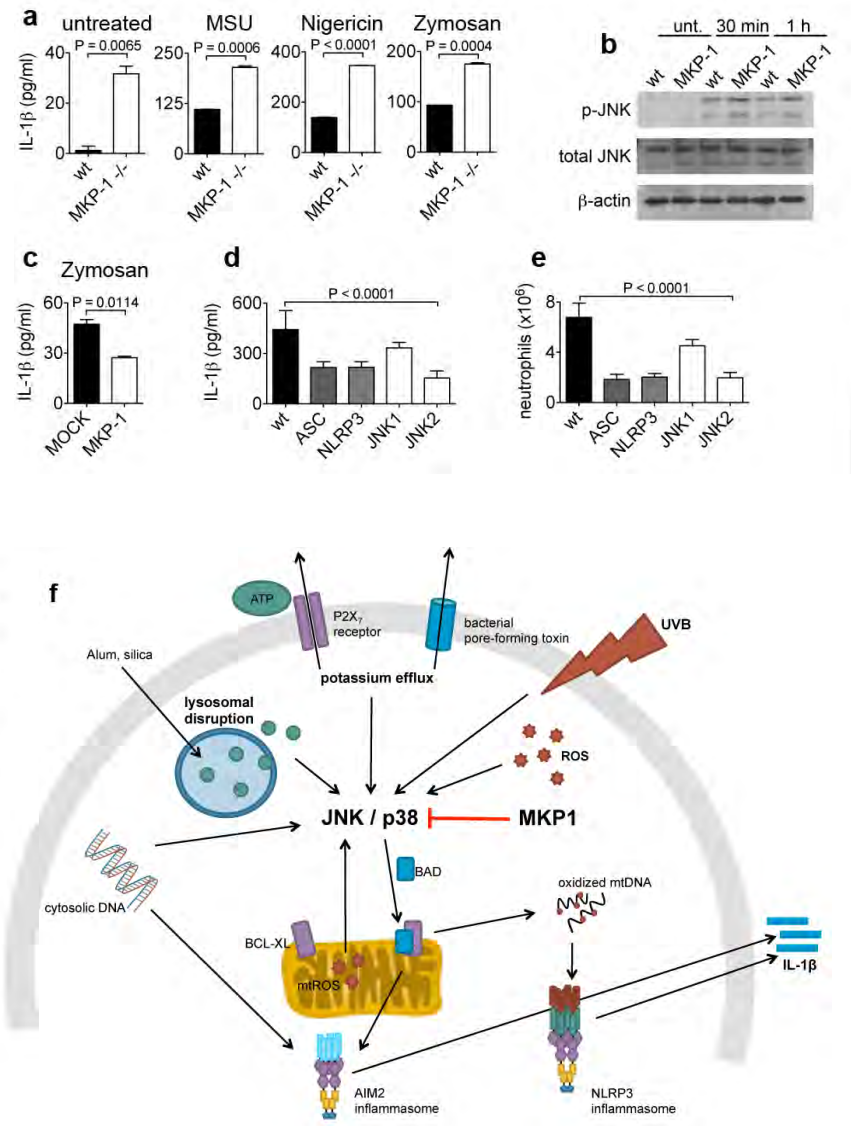

Figure 4: MKP-1 modulates NLRP3 inflammasome activation and JNK2 is required for IL-1β secretion *in vivo*

a, upLPS-pulsed BMDCs from WT and MKP-1 mice were exposed to the indicated inflammasome activators. IL-1β was analyzed in supernatant after 6hrs by ELISA. **b,** Total JNK (tJNK) and phospho-JNK (pJNK) in lysates of upLPS-pulsed BMDC from WT and MKP-1 mice were analysed by Western blot. **c,** J774.1 macrophages transiently overexpressing MKP-1 were pulsed with upLPS and exposed to zymosan. IL-1β was analyzed in supernatants after 6hrs by ELISA. **d, e,** Mice received MSU intraperitoneally and after 6hrs peritoneal lavage was collected, neutrophils were analyzed by flow cytometry and IL-1β was measured by ELISA. **f,** Hypothetical model of inflammasome activation.

To determine if the MAPK JNK2 regulates NLRP3-inflammasome activation and inflammation in an *in vivo* setting, JNK2-deficient and WT mice were injected intraperitoneally with MSU, causing NLRP3-dependent peritonitis as previously reported ([22]). Peritoneal lavage performed after 6 hours revealed significantly reduced levels of IL-1β and neutrophil infiltration in JNK2-deficient mice which was comparable to the reduction in peritoneal IL-1β and neutrophil infiltration observed in mice deficient in inflammasome components ASC and NLRP3 (Fig. 4d,e).

Taken together, our data reveal a novel role for the MAPKs JNK2 and P38 and to BAD in inflammasome activation and unravel an unexpected molecular pathway linking DAMPs and PAMPs to mitochondrial permeabilization, mtDNA release and subsequent inflammasome activation. The release of mtDNA into the cytosol is a key event preceding inflammasome activation, and this process is driven by the above MAPK which when phosphorylated interact with the Bcl-2 family members Bad and Bcl-xL that are likely the essential mediators causing the disruption of mitochondrial integrity (Fig. 4f). Specifically designing therapeutics targeting JNK2, P38 and BAD may provide new avenues for selective inhibition of inflammasome activity and therapy of autoinflammatory diseases ([23]).

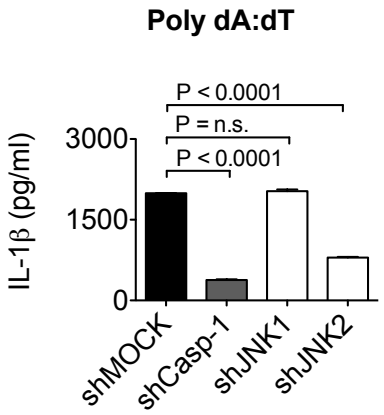

Figure S1: Gene Silencing of JNK2 decreases IL-1β secretion in myeloid cells. THP-1 transfected with shRNA against above genes were differentiated with PMA, pulsed with upLPS and then stimulated with PolydA:dT. Supernatants were analyzed 24h after stimulation by ELISA for IL-1β secretion. Figure is representative of three independent experiments. Statistical significance was determined by Student's t-test.

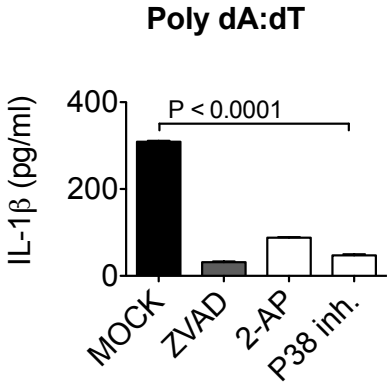

Figure S2: P38 inhibitor decreases IL-1β secretion. HKCs were pre-treated with ZVAD, 2-AP or P38 inhibitor prior exposure with PolydA:dT. Supernatants were analyzed 24h after stimulation by ELISA for IL-1β secretion. Figure is representative of three independent experiments. Statistical significance was determined by Student's t-test.

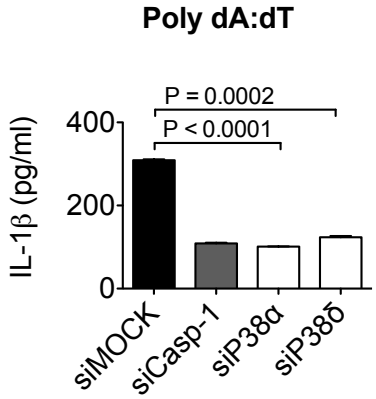

Figure S3: Gene Silencing of P38 decreases IL-1b secretion in keratinocytes. HKCs transfected with siRNA against above genes were exposed to PolydA:dT. Supernatants were analyzed 24h after stimulation by ELISA for IL-1β secretion. Figure is representative of three independent experiments. Statistical significance was determined by Student's t-test.

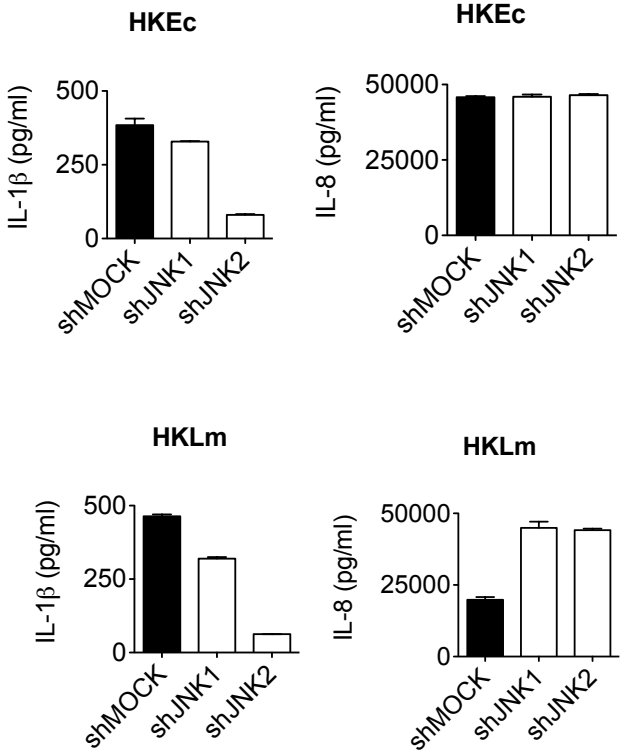

Figure S4: Gene Silencing of JNK does not impair secretion of other cytokines. THP-1 transfected with shRNA against above genes were differentiated with PMA, pulsed with upLPS and then stimulated with heat killed Listeria monocytogenes or heat killed Escherichia coli. Supernatants were analyzed 24h after stimulation by ELISA for IL-8 secretion. Figure is representative of three independent experiments.

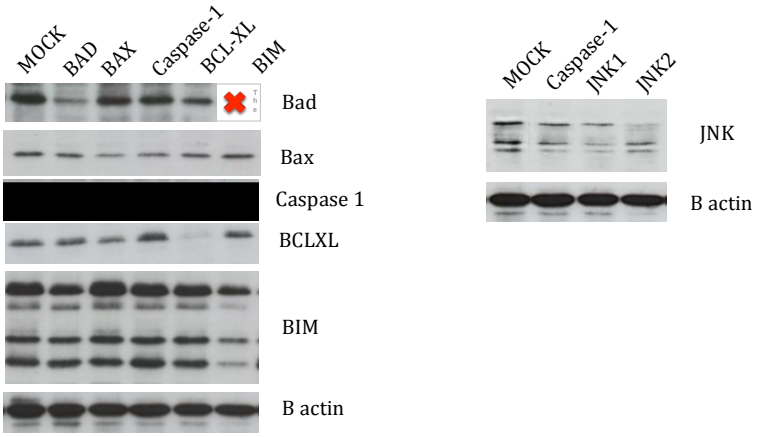

Figure S5: Knock down efficacy of stably transfected shRNA THP1 macrophages. Total cell lysates of THP-1 macrophages transfected with shRNA against above genes were analyzed by western-blot.

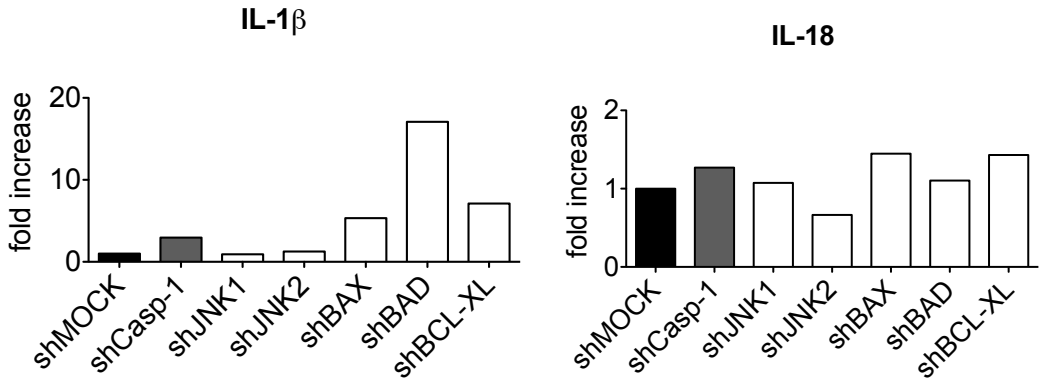

Figure S6: IL-1β and IL-18 expression in stably transfected THP-1 macrophages. THP-1 transfected with shRNA against above genes were differentiated with PMA, pulsed with upLPS and real time pcr was performed for the above genes.

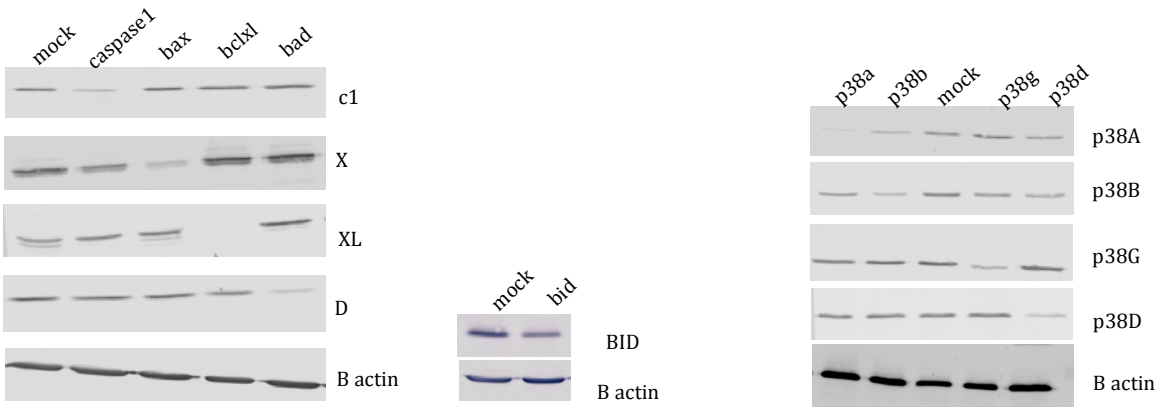

Figure S7: Knock Down efficiency in transiently expressing siRNA Primary Human Keratinocytes. Total cell lysates of primary keratinocytes transfected with siRNA against above genes were analyzed by western-blot.

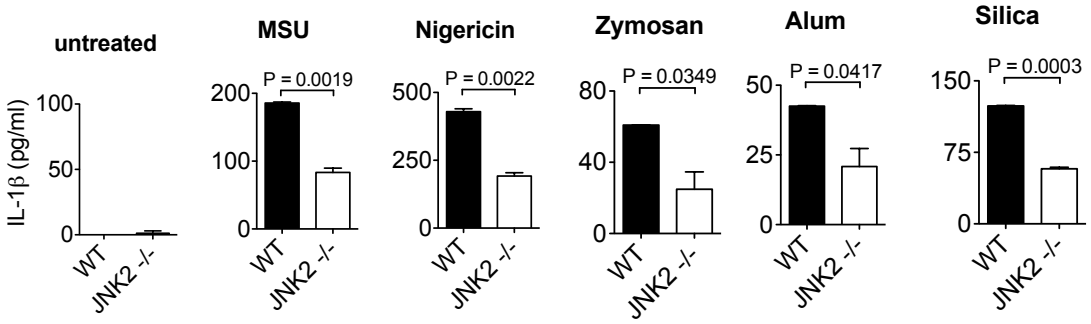

Figure S8: JNK2 KO myeloid cells have impaired IL-1β secretion. BMDCs from WT and JNK2 KO mice were pulsed with upLPS and then stimulated with several NLRP3 inflammasome activators. Supernatants were analyzed 6h after stimulation by ELISA for IL-1β secretion. Figure is representative of three independent experiments. Statistical significance was determined by Student's t-test.

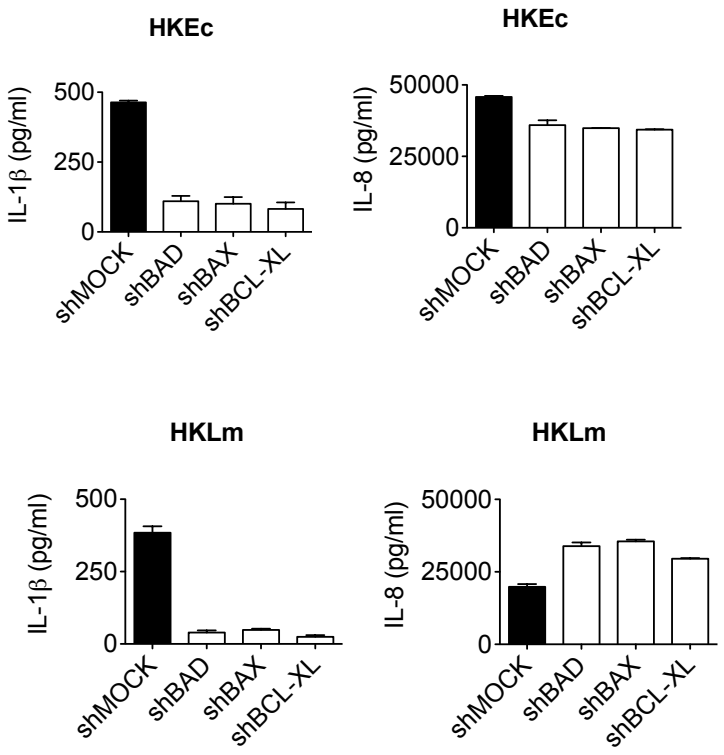

Figure S9: Gene Silencing of BCL2 family members does not impair secretion of other cytokines. THP-1 transfected with shRNA against above genes were differentiated with PMA, pulsed with upLPS and then stimulated with heat killed Listeria monocytogenes or heat killed Escherichia coli. Supernatants were analyzed 24h after stimulation by ELISA for IL-8 secretion. Figure is representative of three independent experiments.

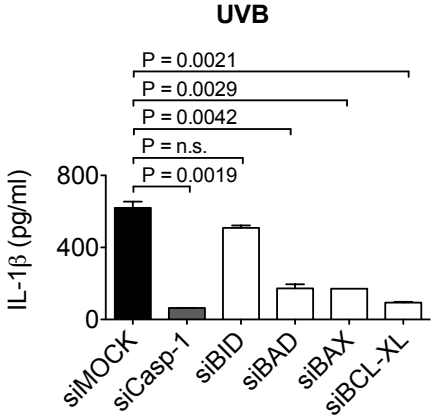

Figure S10: Gene Silencing of BCL-2 family members decreases IL-1β secretion in keratinocytes. Keratinocytes transiently transfected with siRNA against above genes were UVB irradiated. Supernatants were analyzed 6h after irradiation by ELISA for IL-1β secretion. Figure is representative of three independent experiments. Statistical significance was determined by Student's t-test.

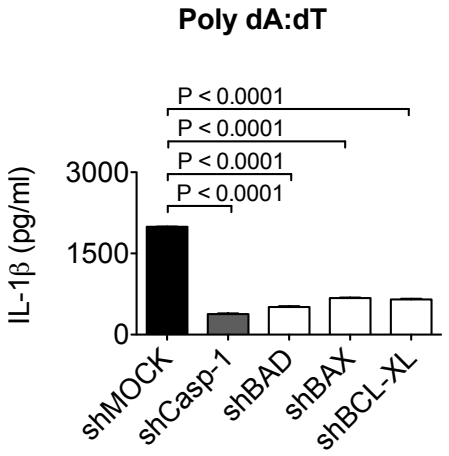

Figure S11: Gene Silencing of BCL-2 family members decreases IL-1β secretion in myeloid cells. THP-1 transfected with shRNA against above genes were differentiated with PMA, pulsed with upLPS and then stimulated with PolydA:dT. Supernatants were analyzed 24h after stimulation by ELISA for IL-1β secretion. Figure is representative of three independent experiments. Statistical significance was determined by Student's t-test.

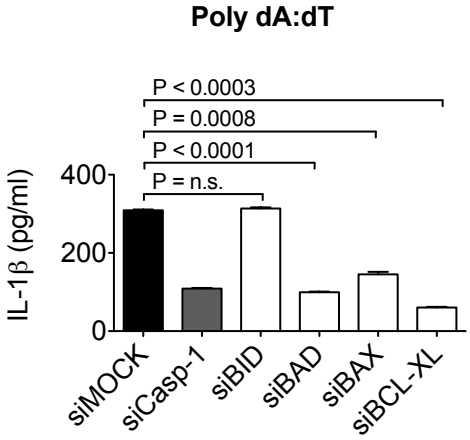

Figure S12: Gene Silencing of BCL2 family members decreases IL-1β secretion in keratinocytes. HKCs transfected with siRNA against above genes exposed to PolydA:dT. Supernatants were analyzed 24h after stimulation by ELISA for IL-1β secretion. Figure is representative of three independent experiments. Statistical significance was determined by Student's t-test.

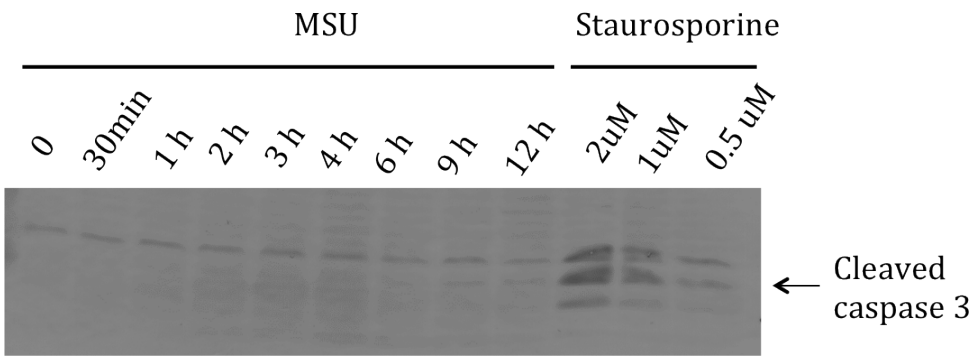

Figure S13: Inflammasome activation does not lead to apoptosis. THP-1 macrophages differentiated with PMA, pulsed with upLPS and then stimulated with MSU (150ug/mL) or staurosporine for 6h. Cell lysates were analyzed by western-blot. Figure is representative of two independent experiments.

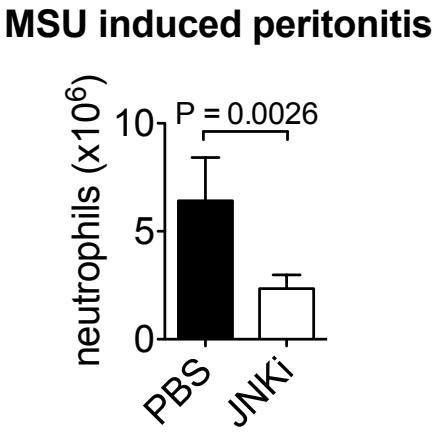

Figure S14: JNK inhibitor decreases MSU induced peritonitis. Mice were treated i.p. with JNK inhibitor 3mg/kg 30min prior i.p. injection of MSU. Six hours after exposition to MSU, peritoneal lavage was analized by FACS. Figure is representative of three independent experiments. Statistical significance was determined by Student's t-test.

Methods

Mice

Nlrp3$^{-/-}$, *Asc*$^{-/-}$ mice were described previously[24,25]. *JNK1*$^{-/-}$, JNK2$^{-/-}$ were obtained from Jackson Laboratories, MKP-1$^{-/-}$, gift from Prof. Kato, Toxicology Department, KIT, Germany. All mice were on a C57BL/6 background.

Cell fractionation

Mitochondrial and cytosolic fractions were isolated from monocytes or PMA-differentiated THP1 cells as previously described[26].

Reagents

Nigericin, rotenone, anisomycin,antimycin, uric acid, ATP, poly(dA:dT) and silica were purchased from Sigma. MitoSOX, Mitotracker green, Mitotracker deep red were from Invitrogen. LDH release kits were from Promega. Ultrapure LPS, Zymosan and R837 were obtained from Invivogen. Inject alum was from Pierce. Anti-human an anti-mouse IL-1β antibodies were from R&D. The anti-NLRP3 antibodies (Cryo-2) were from Adipogen. Anti-caspase-3, BAD, BCL-XL, BAX, BIM, COXIV, JNK, pJNK, P38, YWHAQ, anti-VDAC1 (4866) antibodies were purchased from Cell Signaling. Anti-ASC antibody (AL177) was from Adipogen. Anti-human caspase-1 (sc-622) were from Santa Cruz. Anti-phosphoP38 was from Abcam. All tissue culture reagents were from Invitrogen.

Cell preparation and stimulation

Human THP1 cells were cultured in RPMI 1640 media, supplemented with 10% FBS. THP1 cells were differentiated for 3 days with 100 nM phorbol-12-myristate-13-acetate (PMA) and primed with 100 ng ml^{-1} ultra-pure LPS ON . Bone marrow macrophages were derived from tibia and femoral bone marrow progenitors as described[27], and were primed for ON with 100 ng ml^{-1} Ultra-pure LPS. For the induction of inflammasome activation, 10^6 LPS-primed bone marrow macrophages or PMA-differentiated THP1 cells plated in 12-well plates were treated with MSU (150 μg ml^{-1}), nigericin (15 μM), alum (200 μg ml^{-1}), silica

(200 µg ml^{-1}), R837 (15 µg ml^{-1}) for 6 h or with ATP (5 mM) for 30 min. For poly(dA:dT) transfection, poly(dA:dT) was transfected using Lipofectamine (4 µl ml^{-1}) as per the manufacturer's protocol (Invitrogen). Cell extracts and precipitated supernatants were analysed by immunoblot.

Generation of stable THP1 cells expressing shRNA

THP1 cells stably expressing shRNA were obtained as previously described[27]; shRNA against caspase-1 plasmids has been described[28] and shRNA plasmids against BAD, BCL-XL, BAX, YWHAQ, JNK1,2 were from Sigma.

Generation of human primary keratinocytes transiently expressing SiRNA

Human primary keratinocytes were transfected using INTERFERIN, following manufacturer instructions with 10nM of SiRNA. All SiRNA were from sigma.

Transient transfection of mouse macrophage cell line

J774.1 cells were transfected with 5µg of plasmids and Lipofectamine, following manufactorer instructions. All plasmids were from AddGene.

Confocal microscopy

Human primary keratinocytes on coverslips for 3 days and then used for stimulation or staining with Mitotracker deep red (50 nM). After washing two times with PBST, the cells were fixed with PFA 4% in PBS for 15 min and then washed three times with PBST. After permeabilization with Triton X-100 and blocking with 10% goat serum in PBS, cells were incubated with primary antibodies (in 10% goat serum) at RT for one houre. After washing with PBST, cells were incubated with secondary antibodies (Invitrogen) in 10% goat serum-PBS for 60 min and rinsed in PBST. Confocal microscopy analyses were carried out using a Leica SP5.

Flow cytometric analyses

Mitochondrial mass was measured by fluorescence levels upon staining with Mitotracker green and Mitotracker deep red at 50 nM for 30 min at 37 °C. Mitochondria membrane potential was measured using the kit from Invitrogen and performed according to the manufacturer's instructions. Cells were then washed with PBS solution and re-suspended in cold PBS solution containing 1% FBS for FACS analysis.

ELISA

Cell culture supernatants were assayed for mouse and human IL-1β, IL-8 and TNF-α and mouse-IL1b (R&D) according to manufacturer's instructions.

Statistical analyses

All values were expressed as the mean ± s.e.m. of individual samples. Samples were analysed using unpaired Student's *t*-test.

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Chapter 6

Conclusions and Outlook

During the last decade great progress has been made in understanding the molecular and cellular mechanisms underlying the initiation of inflammatory responses, both beneficial and/or detrimental. Excessive inflammation is a critical detrimental factor in several inflammatory and autoimmune disorders, neurodegenerative conditions, infections, cardiovascular diseases, cerebral ischemia, reperfusion injury, trauma and cancer[1]. A strong link between inflammation and metabolism is becoming increasingly evident and several recent studies have implicated activation of the NLRP3-inflammasome in a variety of metabolic diseases including obesity, atherosclerosis and type-2 diabetes[2,3].

Among cytokines that have proven essential at the onset of inflammation and during the course of an innate immune response is the pro-inflammatory cytokine IL-1β. This cytokine plays a fundamental role in regulating systemic but also certain cutaneous inflammatory responses. Since IL-1β is a very potent pro-inflammatory cytokine, acting locally at extremely low concentrations, and an excess of IL-1β production may have detrimental effects, the activity of the inflammasomes has to be tightly regulated. The mechanisms of such a regulation remain incompletely understood. Recently acquired knowledge regarding the processing and activation of IL-1β has taught us that it is implicated in the pathogenesis of an emerging family of autoinflammatory diseases and fever syndromes[4,5].

Various danger signals activate the NLRP3 inflammasome. These include whole pathogens, pathogen-associated molecular patterns (PAMPs), pathogen-associated molecules (such as bacterial pore-forming toxins and the malaria parasite product haemozoin), environmental irritants (such as

asbestos and UVB irradiation) and damage-associated molecular patterns (DAMPS). The mechanisms by which these structurally distinct molecules trigger NLRP3 oligomerization and inflammasome activation are currently unclear and have been intensely debated in recent literature[6-15]. However, all of the proposed models agree that cytoplasmic K^+ concentration crucially affects inflammasome activation. A second proposal suggests that reactive oxygen species (ROS) account for Nlrp3 activation. This notion is based on the observation that all Nlrp3-activating molecules, such as ATP, nigericin, alum, and uric acid, induce ROS production in macrophages and monocytes. A third model proposes that phagosomal destabilization and cytosolic release of lysosomal cathepsins drive Nlrp3 activation[15] Phagocytosis of crystalline and particulate molecules may cause damage to the lysosomal membrane, which consequently leads to leakage of lysosomal cathepsins into the cytosol. Furthermore, oxidized mitochondrial DNA was proposed as a direct NRLP3 ligand, being released in the cytosol after mitochondrial damage[16-18].
Intracellular calcium concentration was also proposed as a novel mechanism involved in NLRP3 activation[7]. Noteworthy, none of the direct NLRP3 inflammasome activators was shown to be direct ligands of NLRP3.

The present work was aimed at further deciphering the complex mechanisms involved in the sensing of danger by the innate immune system in tissues resulting in IL-1β secretion, extending our understanding of the role of IL-1β in the skin diseases acne and contact hypersensitivity .

The surprising finding reported in chapter 4 of this thesis, showing that IL-1β can turn an almost innocuous molecule as DNTB to a sensitizer hapten, and promote Th-17 differentiation in CHS could have substantial implications in the development of new therapies for diseases in which the immune system is not able to mount an appropriate response, such as cancer, for example, or for situations in which foreign bodies remain invisible to the immune system. This has also implications in is the so-called immunogenic cell death, a situation in which dying cells provide alarm signals to their host. Such dying cell-derived signals could be used in cancer treatment therapies for their

adjuvant properties[19]. Moreover, such a property of IL- 1β could help to understand the pathogenesis of auto-immune diseases[20], where for unknown reasons, the immune system mounts responses against self-antigens, therefore triggering an inappropriate inflammation, injurious to several organs.

Propionibacterium acnes is the most frequent commensal found in inflammatory acne lesions[21-23]. However, a direct role of this bacterium in the pathogenesis of this very common skin disease is a matter of debate. In chapter 3 presented in this thesis, we provide evidence that *Propionibacterium acnes* is able to activate the inflammasome upon internalization of the bacteria by innate immune cells, inducing reactive oxygen species generation, phago-lysosome disruption and leakage of cathepsin B. The NLRP3 inflammasome is responsible for neutrophilic infiltrate *in vivo* and the targeting of IL-1β efficiently prevented the inflammation induced by Propionibacterium acnes.

Although acne vulgaris is a multifactorial complex disease, our observation that the NLRP3 inflammasome and subsequent IL-1β activation are pivotal events in the innate immune response to P. acnes is an important finding that may have implications in the development of new inflammatory agent-based therapies. This brings the focus of the pathogenesis of acne back to inflammation, and provides the opportunity for the development of therapies which are less toxic and have less side effects than the commonly used systemic drugs including retinoids or antibiotics.

Signal transduction in response to environmental stress is a fundamental process in all cells[24]. The mitogen-activated protein kinases (MAPKs) and their downstream targets are one of the essential signaling modules that convert environmental inputs into a plethora of cellular programs[25,26]. In chapter 5 of this thesis, we provide evidence that MAPK pathways control inflammasome activation, which can be modulated by MAPK phosphatase-1. It is known that IL-1β activates the MAPKs JNK and P38, suggesting a positive feedback loop. We observed that JNK2 is required in myeloid cells whereas P38 is required in keratinocytes, indicating a cell population specific mechanism of sensing danger. In the absence of JNK, we

detected less IL-1β secretion upon exposure to a broad range of inflammasome activators, including monosodic uric acid crystals, nigericin (pore forming toxin), alum (vaccine adjuvant), silica crystals, zymosan (yeast wall particles), imiquimod (Toll-like receptor agonist), heat-killed bacteria (E. *coli* and L. *monocytogenes*), ATP and lastly the JNK activator anisomycin. The reduced amount of IL-1β secreted upon JNK activation correlated with reduced loss of mitochondrial membrane potential and reduced leakage of mitochondrial DNA to the cytosol, considered to be, when oxidized, a direct ligand of NLRP3.

Notably, we could demonstrate that BCL-2 family members, which have largely documented roles in cell death and emerging roles in cell metabolism and mitochondria physiology, also play a key role in inflammasome activation. We showed, in myeloid cells and keratinocytes, that the absence of BAD, BAX and BCL-XL led to reduced mitochondrial damage upon inflammasome activation accompanied by a reduced release of mitochondrial DNA into the cytosol. As a direct consequence of this, IL-1β secretion was impaired, upon stimulation with a broad range of inflammasome activators.

Despite the considerable advance in our understanding of the regulation of inflammasome activation resulting from our discovery of the key role of JNK and P38, the events linking the encounter of danger and/or pathogens and MAPK activation still remain to be elucidated. Notably, whether NLRP3 is a direct target of MAPK or if NLRP3 has other possible endogenous ligands (such as cytochrome c or other mitochondrial proteins) is not known to date. In addition to mitochondrial damage-related events, other, intracellular danger signals may be involved in inflammasome activation, as recently reported for calcium and ER stress[7,27,28]. The understanding of these mechanisms linking cell injury and inflammation could help us to understand the possible mechanisms involved in the pathogenesis of several auto-immune and auto-inflammatory diseases. JNK was shown to play a very important role in many chronic diseases that are associated with chronic inflammation, as hypertension, artherosclerosis, obesity and insulin resistance, and classic chronic inflammatory diseases as diabetes, rheumatoid arthritis[29-35]. Recently it was suggested that the inflammation in

these diseases is dependent of NLRP3 inflammasome[36-41]. We propose that, among other pro-inflammatory functions, JNK has also the regulation of NLRP3 activation, providing novel targets for treatment of a broad range of diseases in which IL-1β is involved.

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Appendix A

Abbreviation and units

A.1 Abbreviations

ANOVA	analysis of variance
ASC	apoptosis-associated speck-like protein containing a CARD
ATP	adenosine triphosphate
BAD	bcl-2 associated death promoter
BAX	bcl-2 associated X protein
BCL-XL	b-cell lymphoma extra large protein
BID	bh-3interacting domain death agonist
BIM	bcl-2 interacting mediator of cell death
BPE	bovine pituitary extract
BSA	bovine serum albumin
CARD	caspase recruitment domain
Co-IP	co-immuno precipitation
Ctr	control
DAP	death-associated protein
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ECL	enhanced chemoluminiscence
EDTA	ethylediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
ER	endoplasmitic reticulum
ERK	extracellular signal regulated kinase
FCS	fetal calf serum
FITC	luorescein isothiocyanate

HRP	horseradish peroxidase
IPAF	inflammatory protease activating factor
IFN	interferon
IKB	inhibitor of nuclear factor kb
IKK	inhibitor of nuclear factor kb kinase
IL	interleukin
IL-1R	interleukin-1 receptor
IL-1Ra	interleukin-1 receptor antagonist
IP	immune precipitation
JNK	C-Jun N-terminal Kinase
KGF	Keratinocyte growth factor
LB	Luria-Bertani
LDH	lactate dehydrogenase
LPS	lipopolysacharide
LRR	leucine rich repeat
MAP	mitogen activated protein
NLRP	NACHT, LRR and PYD domains-containing protein
NAIP	NLR family apoptosis inhibitory protein
NFKB	nuclear factor kB
Ns	non significant
O/N	over night
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT	room temperature
shRNA	short hairpin ribonucleic acid
siRNA	small interference ribonucleic acid
SDS	sodium dodecyl sulfate
SFM	serum fee medium
SN	supernatant
TLR	toll-like receptor
TNF	tumor necrosis factor
UV	ultraviolet light
UVB	ultraviolet B
VEGF	vascular endothelial growth factor

WT	wild-type

A.2 Units

bp	base pairs
°C	Celsius degrees
cm	centimeter
Da	Dalton
dpi	dots per inch
g	gram
µg	microgram
ng	nanogram
pg	picogram
h	hour
J	Joule
µL	microliter
mL	milliliter
M	molar
uM	micromolar
nM	nanomolar
U	Units

Appendix B

Publications

2013 Gehrke S, Fenini G, Contassot E, French LE. Novel Role for JNK and P38 in inflammasome activation- manuscript in preparation

2013 Gehrke S, Huber R, Fenini G, Kerl K, Kleiber I, Jankovic D, Contassot E, French LE. Necrotic melanoma cells induce IL-1β secretion of infiltrating antigen-presenting cells via activation of the NLRP3 inflammasome- manuscript in preparation

2013 Kistowska M, Gehrke S, Jankovic D, Kerl K, Fetteschoss A, Contassot E, French LE. Sensing of *Propionibacterium acnes* by the NLRP3-inflammasome drives inflammation in acne - submitted

2011 Contassot E, Jankovic D, Schuler P, Preynat-Seauve O, Gehrke S, Kerl K, Beermann F, French LE. Carcinogen treatment in mouse selectively expressing activated N-Ras(Q61K) in melanocytes recapitulates metastatic cutaneous melanoma development. Pigment Cell Melanoma Res. 2011 Nov 29 Mar;25(2):275-8.

2010 Lockwood LL, Gehrke S, Navarini AA. Dermoscopy of Pited keratolysis. Case Rep Dermatol. 2010 Aug 19;2(2):146-148.

2008 Watanabe H, Gehrke S, Contassot E, Roques S, Tschopp J, Friedmann PS, French LE, Gaide O. Danger signaling through the inflammasome acts as a master switch between tolerance and sensitization. J Immunol. May 1;180(9):5826-32

Appendix C

Curriculum Vitae

1. PERSONAL INFORMATION

Surname: **GEHRKE**

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2. AREAS OF INTEREST

RESEARCH

Immunology with focus on NOD-like receptors, Inflammasomes and their role in immune regulation, innate immunity and cancer.

Translational Research in animal models

COMMERCIAL

Administration, accounting and human resources

3. EDUCATION

Actual:

PhD in Immunology/Molecular Biology
University of Zurich
University Hospital Zurich

Superior Studies

2000 – 2007 Medicine degree
Health Sciences Sector and Biological Sciences Sector of Parana Federal University (*Universidade Federal do Paraná)*
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Training Courses

2001 Training in Cell Biology, at the Cell Biology Department of Biological Sciences Sector, *Universidade Federal do Paraná*, at CURITIBA-PR (Brazil).

2001-04 Research training in Cell Biology, at the Inflammation and Neoplasic Cells Laboratory Cell Biology Department of Biological Sciences Sector, *Universidade Federal do Paraná*, at CURITIBA-PR (Brazil).

2003-04 Research training in Cell Biology and Medical Practice, at the Home Based Care Gabane and Francistown, The Health Ministry of Botswana, Botswana, Africa.

2003 Training at the Emergency Service at "*Hospital do Trabalhador*" Hospital, CURITIBA-PR (Braziil).

2006 Internship in Surgery and Clinical Surgery, at Clinics University Hospital (*Hospital de Clínicas*), *Universidade Federal do Paraná*, CURITIBA-PR (Brazil).

2006 Internship in Paediatrics at Clinics University Hospital (*Hospital de Clínicas*), *Universidade Federal do Paraná*, CURITIBA-PR (Brazil).

2006 Internship in Gynaecology and Obstetrics at Clinics University Hospital (*Hospital de Clínicas*),and Victor Ferreira do Amaral Maternity Hospital, *Universidade Federal do Paraná*, CURITIBA-PR (Brazil).

2006 Optional training in Gynaecology and Obstetrics at Clinics University Hospital (*Hospital de Clínicas*), *Universidade Federal do Paraná*, CURITIBA-PR (Brazil).

2006 Internship in Internal Medicine at Clinics University Hospital (*Hospital de Clínicas*), *Universidade Federal do Paraná*, CURITIBA-PR (Brazil).

2007 Optional Internship in Dermatology, at Clinics University Hospital (*Hospital de Clínicas*), *Universidade Federal do Paraná*, CURITIBA-PR (Brazil).

2007 Optional Internship in Dermatology Research at Centre Medical Universitaire (CMU) , Faculté de Medecine de l'Université de Genève (UNIGE), Genève (Switzerland).

High School

1999 *Colégio Positivo*
CURITIBA – PR (BRAZIL)

1997 – 98 *Colégio Franciscano Santo Antônio*
BLUMENAU-SC (Brazil)

SCHOLARSHIPS

2008-13 PhD Student, Swiss National Found (SNF) granted.

2002-03 CAPES (Superior Education Personnel Development Program Coordination) / PIBIC (Scientific Scholarship Institutional Program) / CNPQ (National Council for Scientific Development). At the Inflammation and Neoplasic Cells Laboratory Cell Biology Department of Biological Sciences Sector, *Universidade Federal do Paraná*, CURITIBA-PR (Brazil).

2003 National Treasury/ Parana Federal University Fund. Development, structuration and execution of the Extention Course : Culture and Medicine : Philosophy and Emancipation, the first Philosophy course to Medicine students of the Parana Federal University. Pathology Department, Health Sciences Sector and the Philosophy Department of the Human Sciences. *Universidade Federal do* Paraná, CURITIBA-PR (Brazil).

2001 National Treasury/Parana Federal University Fund. Instructorship at the discipline of Cell Biology, Cell Biology Department at Biologic Sciences Sector, *Universidade Federal do Paraná*, CURITIBA-PR (Brazil).

4. ACADEMIC ACTIVITIES

2003 Director of the Medical Education Department of the Medical Students Academic Centre of the Parana Federal University, CURITIBA-PR (Brazil)

2004 Academic representative at the First Regional Health Secretary of Parana State, at Paranagua, through the Community Health Department, Health Sciences Sector, *Universidade Federal do Paraná*, à CURITIBA-PR (Brésil).

5. SCIENTIFIC PRODUCTION

PUBLICATIONS

2013 Gehrke S, Fenini G, Contassot E, French LE. Novel Role for JNK and P38 in inflammasome activation- manuscript in preparation

2013 Gehrke S, Huber R, Fenini G, Kerl K, Kleiber I, Jankovic D, Contassot E, French LE. Necrotic melanoma cells induce IL-1β secretion of infiltrating antigen-presenting cells via activation of the NLRP3 inflammasome- manuscript in preparation

2013 Kistowska M, Gehrke S, Jankovic D, Kerl K, Fetteschoss A, Contassot E, French LE. Sensing of *Propionibacterium acnes* by the NLRP3-inflammasome drives inflammation in acne - submited

2011 Contassot E, Jankovic D, Schuler P, Preynat-Seauve O, Gehrke S, Kerl K, Beermann F, French LE. Carcinogen treatment in mouse selectively expressing activated N-Ras(Q61K) in melanocytes recapitulates metastatic cutaneous melanoma development. Pigment Cell Melanoma Res. 2011 Nov 29.

2010 Lockwood LL, Gehrke S, Navarini AA. Dermoscopy of Pited keratolysis. Case Rep Dermatol. 2010 Aug 19;2(2):146-148.

2008 Watanabe H, Gehrke S, Contassot E, Roques S, Tschopp J, Friedmann PS, French LE, Gaide O. Danger signaling through the inflammasome acts as a master switch between tolerance and sensitization. J Immunol. May 1;180(9):5826-32

POSTERS IN CONGRESS

2010 *Necrotic tumor cells induce inflammasome activation*. S. GEHRKE, M.KISTOWSKA, D. JANKOVIC, M. SPALINGER, R. DUMMER, E. CONTASSOT, L.E. FRENCH. European Society for Dermatological Research Meeting,8-10 September 2010 Helsinki (Finland)

2008 *The inflammasome regulates antigen presenting cell trafficking in the skin. S*. GEHRKE, H. WATANABE, S. ROQUES, A. CAILLON, L. E. FRENCH, O. GAIDE. International Investigative Dermatology Meeting,14-17 May 2008, Kyoto (Japan)

2007 *Eyebrows Alopecia: A Diagnostic Exercise*. F MULINARI-BRENNER, PL CHIMINACIO, S GEHRKE. XXI *World Congress* of *Dermatology* , September 30-October 5, 2007 Buenos Aires (Argentina)

2006 *Improved Quality of Life in HIV/AIDS Patients Treated with Canova en Botswana.* DI BERNARDI, R. ; GEHRKE, S. ; CASAROTTO, P. ; REDE, K.S. ; BUCHI, E.A. ; BUCHI, D.F. *Improving the Success of Homeopathy 5, The Royal London Homoeopathic Hospital.* 26 – 27 January 2006. LONDON (UK)

2004 *Evaluation of the effects of Canova Medicine in HIV+/AIDS patients quality of live at Botswana, Africa*. DI BERNARDI, R.P. ; GEHRKE, S. ; CASAROTTO, P ; BUCHI, E ; BUCHI, D.F. VIII National Symposium and VIII International Meeting of Institutional Research in Homeopathy. 20 – 22 May. SÃO PAULO-SP (Brazil)

2003 *Immunomodulation in Sarcoma-180 Bearing Mice.* WAL, R. ; SATO, D.Y.O. ; OLIVEIRA, C.C. ; LOPES, L. ; OLIVEIRA, S.M. ; DI BERNARDI, R.P. ; GEHRKE, S. ; PALAURO, F. ; BUCHI, D.F. *Cell and Molecular Biology of Cancer*. ISREC. 22 – 25 January. LAUSANNE (Switzerland)

2002 *The Effects of a Homeopathic Medecine on Mouse Macrophages.* OLIVEIRA, C.C. ; LOPES, L. ; GODOY, L. ; OLIVEIRA, S.M. ; DI BERNARDI, R.P. ; GEHEKE, S. ; PALAURO, F. ; BUCHI, D.F. *47nd American Society for Cell Biology Annual Meeting.* 14 – 18 December. SAN FRANCISCO-CA (USA)

2002 *The Action of the homeopathic medicine Canova Method in mice peritoneal macrophages*. GEHRKE, S.; OLIVEIRA, C.C. ; LOPES, L. ; BUCHI, D.F. Xth Scientific Initiation Congress EVINCI, at the *Universidade Federal do Paraná.* CURITIBA-PR (Brazil)

2001 *Nitric Oxide Generation by Mouse Peritoneal Machrophages Treated with Método Canova*. GODOY, L ; SATO, D.Y.O. ; WAL, R ; OLIVEIRA, C.C. ; LOPES, L ; GEHRKE, S. ; BUCHI, D.F. *XVII*I *Congress of the* Brazilian Society for Microscopy and Microanalysis ; 28 – 31 October. AGUAS DE LINDOIA (Brazil)

6. LANGUAGE SKILLS

Mother tongue: Portuguese

Foreign Languages: English (advanced level), French (advanced level), Spanish (advanced level) and German (intermediate level).

7. COMPUTER SKILLS

Microsoft Office (advanced), Graphpad Prisma, FlowJow, BD FACS systems.

8. PERSONAL DEVELOPMENT

Tenor (sang: Bastien und Bastienne, Die ZauberFlöte, Canzoni Napoletane, Don Giovanni);

Co-Founder of two NGOs: Instituto Arapoty (Sao Paulo, Brazil. Works with ancient culture, native brazilian culture and traditional medicine, environmental conscience – water resources- collaboration with France Libertés-Danielle Mitterand) and Accorde (Curitiba, Brazil, Bringing culture and developing trans-disciplinarity skills through the music);

Humanitarian Work with HIV patients in Sub-Saharan Africa, Creating a new system for treating patients in precarious conditions with not enough health professionals – formation of community leaders for health caretakers;

Sports: Rowing, tennis (passions) and swimming.